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Mast Cells are important yet poorly understood components of the immune system. Study of mast cells has been hampered by difficulty in obtaining primary cells, the lack of functional cell lines and the high degree of dissimilarity between human and murine mast cells. Adipose derived stem cells are abundant cells in the human body that are easily and relatively non-invasively obtained. Using media containing mediators secreted by skin derived mast cells, adipose derived stem cells were differentiated into functional mast cells. Mast cells differentiated from adipose derived stem cells were tested for their ability to respond to and kill cancer cells using an IgE-mediated targeting method. Mast cell induced cytotoxicity was observed as well as interactions between mast cells and cancer cells. Adipose derived mast cells may represent a platform for allergo-oncology.

DIFFERENTIATION OF FUNCTIONAL HUMAN MAST CELLS FROM ADIPOSE DERIVED STEM CELLS AND THEIR APPLICATION IN ALLERGO-ONCOLOGY AND THE TREATMENT OF BREAST CANCER

by

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Approved by

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DEDICATION

To my grandfather, Francis Tromba

APPROVAL PAGE

This dissertation written by Jesse David Plotkin has been approved by the following committee of the Faculty of the Graduate School at the University of North Carolina at Greensboro.

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CHAPTER I

INTRODUCTION

Mast cells (MCs) are important immune cells responsible for coordinating immune responses such as hypersensitivity, inflammation as well as playing roles in wound healing, adipose homeostasis and nociception. Despite being identified well over one hundred years ago, MC biology remains shrouded in mystery and debate due to the difficulty in obtaining them.

Since their discovery over one hundred years ago, the association between MCs and cancer has been well documented and poorly understood. The association of MCs with solid tumors has been appreciated since the initial description of MCs in the nineteenth century, indeed Paul Ehrlich himself noted that MCs were commonly associated with carcinomas [1] as well as being found by others soon after to be associated with all tumor types investigated. [2]. Debate still surrounds the effect of MCs on tumors, and whether they contribute to tumor and disease progression or whether they constitute part of the immune systems efforts to clear the body of its tumor burden. Evidence is mounting, however, that the effect of MCs on cancer is highly context dependent, with the nature, location, and mutational burden of the tumor as well as the type of MC present, its location within the tumor and the overall inflammatory state of the individual bearing the tumor [3]. Due to their highly pleiotropic nature, it is not surprising that MCs are not homogeneous in the role in cancer biology and care must be

taken when considering their use as targets in cancer therapies. While the exact nature of the association between MCs and cancer remains a topic of debate, studies correlating the presence of MCs in tumors with patient survival outcomes have identified certain types of malignancies wherein the presence of MCs seems to be favorable to survivability [4]–[6]. One of these types of cancer is invasive, HER2 overexpressing breast cancer [7]–[9].

The study of MCs and their involvement in cancer is especially difficult given the highly heterogeneous and context specific nature of both MC function and cancer biology. There is clear evidence for highly detrimental effects of MCs on cancer survival and clear protective effects as well. Given the confluence of factors precluding the study of MCs in the laboratory, it is hardly surprising, however still somewhat infuriating, that they are given short shrift in the field of immuno-oncology. The introduction of a suitable source of functional human MCs is essential to understanding and harnessing the full potential of MCs in human biology.

Allergo-oncology refers to the use of IgE to target Fc receptor-bearing cells to tumors for the purpose of killing cancer cells in patients. Allergo-oncology is a new approach in immunotherapy that relies on the use of IgE rather than IgG as its targeting molecule. While there exist, many FDA approved monoclonal antibody therapies, all are of the IgG class of immunoglobulins. There are advantages and disadvantages to the use of monoclonal IgG-based drugs. The chief disadvantage is danger of triggering the patients' immune system to produce off-target cell, tissue, organ and ultimately system damage which in extreme cases can be fatal. Adipose tissue is being used as a source of adult stem cells with the goal of providing the MC research community with an

accessible source for cells with which to do research. The purpose of the present study was to investigate the viability of using ADMC as a tool for allegro-oncology. In order to assess the ability of ADMC to induce cytotoxicity, two mechanisms were investigated, Antibody Dependent Cell induced Cytotoxicity (ADCC) and Mediator Induced Cell Killing (MICK). Preliminary studies of ADMC-induced cytotoxicity of breast cancer cells *in vitro* are promising and provide a foundation for future animal studies.

CHAPTER II

REVIEW OF THE LITERATURE

II.1 Introduction to Mast Cells (MCs)

In the late 19th century, German microscopist Paul Ehrlich described a type of cell in frogs that was identified by the presence of numerous metachromatic granules. Ehrlich named these cells "*mastzellen"* derived from the German word "*mast"* meaning fattening or feed [2], [10]. Found in almost all tissues in the human body, mast cells (MCs) play an important role as sentinel cells, sensing and alerting other immune cells to the presence of pathogens, particularly parasites. The functions of MCs are diverse and include protection against pathogenic infection and coordinating inflammatory actions of effector cells in response to foreign bodies. MCs are unique among the immune cells in that they participate in both innate and adaptive immunity. In addition to their roles in protection from pathogens, MCs serve other important roles in maintaining normal human physiology including in wound healing, angiogenesis [10], and adipocyte maturation [11]. While the body of knowledge surrounding MC biology is growing, it is truly dwarfed when compared to that of other immune cells. The vast differences in phenotype and function between human and rodent MC, human MC lines, and MC obtained from tissue sources (e.g. cord blood) has severely hampered basic research on MC biology [12][13] and is a major impediment to understanding the full role played by MCs in health and disease.

II.2 Mast Cell Biology

II.2.i Mast Cell Development

The exact nature of MC differentiation in humans remains unclear and while the mechanisms of murine MC differentiation are better understood, they are unsatisfactory when attempting to understand the analogous human biology. What is known is that MCs like all other hematopoietic cells, arise from hematopoietic stem cells (HSC) in the bone marrow [14]. This was demonstrated using a line of beige mice, which exhibit giant MC granules and can thus be used as a phenotypic marker, as donors to replace bone marrow of an irradiated wild-type mouse [15] and showing that giant granule MCs were subsequently observed in the tissues of the irradiated mice. This was later demonstrated to also be true in humans using *ex vivo* culture of human bone marrow cells [16]. Hematopoiesis is divided into two lineages, lymphoid and myeloid, of which MCs belong to the myeloid lineage, arising from the common myeloid progenitor (CMP). How many steps separate the CMP from the mast cell progenitor (MCP) is not known.

MC progenitors leave the bone marrow and enter the blood stream as agranular, $CD34^{+}$, KIT^{+}, cells [17][18]. A more refined definition of the immature circulating progenitor cell that gives rise to tissue MCs is lacking. It has recently been suggested, however, that the circulating progenitor may be a relatively rare population of Lin^{-} $CD34^{hi}$ $CD117^{int/hi}$ $Fc\varepsilon RI^{+}$ cells [19]. While this is an exciting prospect, more research will be required to determine whether this population is in fact the immature, circulating form of tissue MCs.

MCP exit the blood stream after an indeterminate period of time and enter tissues where they receive cues from the local microenvironment to complete their differentiation into mature MCs. It is thought that the major chemotactic signal for MCs to enter tissues is stem cell factor (SCF) which is a 245 amino acid protein with a membrane-bound and a secreted form, both of which are ligands of c-KIT. Other chemokines may also be involved, as there is evidence from mouse studies that disruption of some chemokines result in aberrant MC homing [20], but to a lesser extent than SCF. SCF serves multiple functions in the progression from circulating progenitor to fully differentiated tissue MC. In addition to providing a chemotactic signal, SCF also regulates differentiation and maturation of the immature progenitor. c-KIT is a receptor tyrosine kinase, upon binding of SCF to the extracellular domain of c-KIT induces receptor dimerization which allow the catalytic, cytoplasmic domains to interact and cross-phosphorylate multiple tyrosine residues. These phosphorylated cytoplasmic domains then serve as binding sites for various other proteins via either an Src homology-2 or phosphotyrosine-binding domain [20]. A large number of signaling proteins are able to bind to activated c-KIT, belonging to several different signaling pathways [21], including the phosphatidylinositol-3 kinase, protein kinase C and phospholipase C pathways, all of which lead ultimately to phosphorylation cascades terminating in the regulation of gene expression. Other pathways that can be induced through c-Kit signaling are the Jak/Stat and the Ras/MAPK pathways [20]. Activation of these signaling pathways give the immature MC progenitors cues to begin terminal differentiation and to begin to localize to tissues. After receiving the appropriate signals

for tissue infiltration and differentiation MCs must exit the blood stream and infiltrate tissues such as skin, gut and lung mucosa or any sites of inflammation [22][23]. To achieve tissue infiltration MCs must first interact with vascular endothelial cells which they do via a combination of cell surface adhesion molecules [24]. Human MC progenitors produced by *in vitro* differentiation of cord blood were investigated for their ability to bind human umbilical vein endothelial cells under physiological flow and in the presence of antibodies for different adhesion molecules. In the context of this assay, these MC progenitors were found to have binding inhibited most robustly by antibodies blocking their interaction with α_4 integrin, vascular adhesion molecule-1 and P-selectin glycoprotein ligand-1. In addition to these adhesion molecules, some participation was also indicated in other molecules such as integrin β_1 and integrin β_7. As well as their normal migration into tissues as part of their maturation, MCs also respond to chemotactic signals originating from sites of inflammation or injury.

II.2.ii Mast Cell Granules

MC granules are a class of secretory granules (SG) containing preformed immune mediators [25]. Due to their low pH, the presence of lysosomal enzymes, and the fact that they undergo various other lysosomal functions they are termed lysosome-related organelles. MC granules contain a complex and changing complement of factors whose exact make up depends upon factors such as anatomical location, disease state and presence of xenobiotics. The contents of MC granules can be divided into five major categories: 1) enzymes - predominantly proteases and β-hexosaminidase but also

including peroxidase, phospholipase and matrix metalloproteinases, 2) biogenic amines – histamine and serotonin, 3) cytokines, 4) proteoglycans – heparin and chondroitin and 5) peptides [26]. Tryptase and chymase, two serine-class peptidases, are perhaps the most widely studied components of MC granules and are the basis of identification of the two major subtypes of human MCs, tryptase-positive (MC_T) cells which are found predominantly in the gut mucosa and tryptase/chymase-positive (MC_{TC}) cells which are found in the skin [27].

Tryptases are a diverse group of enzymes characterized by their preferred substrate, the peptide bond following lysine and arginine found in polypeptides. In humans, there are at least six distinct forms of MC tryptase, two of which are inactive, arising from four genes, among these six, five are secreted and one is membrane bound. There are also several pseudogenes in the human genome that no longer produce functional proteins. In MC granules, tryptases are the most abundant proteins, existing in oligomeric structures similar to proteasomes. These structures when released into the bloodstream are highly resistant to peptidase inhibitors such as α_1-anti-trypsin and α_2-macroglobulin and may be stabilized by proteoglycans also found in MC granules [27]. Chymase is a chymotryptic peptidase found in human MCs, characterized by its preferred substrate of the peptide bond following tyrosine and phenylalanine. As mentioned previously, chymase is not expressed in all MCs, predominating in cells found in the lung and other mucosal tissues. MC chymase arises from a single locus and is present in only one form. Chymase is expressed almost exclusively in MCs, however expression has also been reported in smooth muscle cells. Cathepsin G is another chymotryptic peptidase

found in MC SG. It is characterized by a preference for substrates containing tyrosine and phenylalanine as well as amino acids with basic side groups, meaning it also has tryptic peptidase-like features. Cathepsin G is not found in MC_T type cells [27].

Another major component of all MC SG is β-hexosaminidase. MCs are peculiar with regards to β-hexosaminidase in that it is found predominantly in the SG as opposed to the lysosome. β-hexosaminidase is a heterodimeric enzyme present in large amounts in lysosomes and in the SG of MCs it is responsible for the cleavage of *N*-acetylhexosamine from *N*-acetyl-β-hexosaminides, which is an essential part of glycoprotein metabolism and therefore general cellular metabolism. The importance of β-hexosaminidase is evidenced by the fact that loss of function results in Sandhoff disease, which has far reaching effects including blindness, psychosis, and hepatosplenomegaly [28], [29]. Furthermore, Fukuishi *et al* showed that ablation of β-hexosaminidase from the MCs of mice prevented clearance of bacteria from the peritoneal cavity [30]. It is hypothesized that β-hexosaminidase along with lysozyme is able to disrupt the peptidoglycans present in gram positive bacteria. β-hexosaminidase is also important for the study of MCs in that it is the most widely used biomarker for MC degranulation. In the eponymously named assay, a substrate containing an *N*-acetylhexosamine (most commonly *N*-acetyl-β-D-glucosamine) is incubated with MC supernatant and a colorimetric change is measured.

Histamine is found in all MC SG, regardless of peptidase content. Histamine is a monoamine neurotransmitter and modulator of vascular tone derived from the amino acid histidine. Histamine in MC SG is associated with heparin.

One of the qualities unique to MCs is the presence of preformed tumor necrosis factor alpha (TNF-α) in their secretory granules [31]. TNF-α is released upon stimulation of MCs and induces the expression of adhesion molecules on endothelial cells. These adhesion molecules are then able to recruit other immune cells associated with the inflammatory response such as neutrophils. It has been shown in a rodent leukemic MC line that a fusion protein of green fluorescent protein and TNF-α localizes to the secretory granule and can be released. It was further demonstrated using inhibitors of glycosylation and the sorting of glycoproteins that this process is partially dependent on the mannose-6-phosphate receptor and the post-translational modification of *N*-glycosylation [32].

II.2.iii IgE Mediated Response

MCs respond to IgE through one of three types of IgE receptor, the high affinity FcεRI receptor, CD23 which is sometimes referred to as the low affinity IgE receptor and galectin-3 [33]. FcεRI is a tetrameric, membrane receptor whose cognate ligand is the Fc domain of IgE. FcεRI is critical for triggering the release of granule contents in response to allergen [34] as well as beginning a series of secondary processes that allow MCs to co-ordinate the inflammatory response. The FcεR is composed of three chains, alpha, beta and gamma, the tetramer being formed by one alpha, one beta and two gamma chains [35]. The ligand binding domain of FcεRI is located at the N-terminal of the alpha chain and is comprised of two immunoglobulin-related domains which bind Cε2 and Cε3

domains in the Fc portion of the IgE molecule. The stoichiometry of binding of IgE: FcεRI is 1:1 and the affinity of the FcεRI for IgE is high, with a K_a on the order of 10^{10} M^{-1} [33]. The fully processed alpha chain is N-glycosylated at seven residues. These residues were shown through sequential mutagenesis of the alpha subunit to be required for proper folding in the endoplasmic reticulum [36]. The function of the cytoplasmic tail of the alpha subunit is poorly understood. The beta and gamma chains are also transmembrane proteins, however they do not participate in ligand binding [37]. Both the beta and gamma chains possess immunoreceptor tyrosine-based activation motifs (ITAMs) which, upon phosphorylation become binding sites for various other signaling molecules bearing a Src homology 2 domain, the most well described are Lyn, which binds ITAMs on the beta subunit and Syk, which binds ITAMs on the gamma subunit, both of which belong to the Src family of kinases. Activation of these signaling molecules can have a wide range of effects depending on the nature of activation including degranulation, secondary cytokine and lipid mediator synthesis, increased survival and increased receptor expression. In the non-ligand bound state, the ITAMs are not phosphorylated and the receptor is not active. In the normal physiological context, tissue MCs are found decorated with IgE bound to FcεRI receptors. When an antigen recognized by IgE molecules on the MCs surface binds two or more IgE molecules, the FcεRI complexes are brought close together into an aggregated state. Upon aggregation Lyn, which has a weak affinity for the ITAM in its unphosphorylated state, is brought in close proximity with the beta chains of adjacent receptors in the aggregate which it then trans-phosphorylates. The phosphorylated beta chain then induces an increase in the

kinase activity of Lyn which leads to the phosphorylation of ITAMs on the gamma subunits of the FcεRI. The phosphorylated gamma ITAMs are then able to recruit and activate Syk molecules. An alternative but very much related pathway for antigen induced FcεRI activation involves a third Src family kinase, Fyn, which like Lyn binds ITAMs on the beta subunit of FcεRI. Fyn is also negatively regulated by activated Lyn.

II.2.iv Non-IgE Mediated Response

In addition to the IgE-FcεRI pathway, MCs can be activated through various other pathways due to the very high number and diversity of receptors expressed of their cell surface [38]. Among the stimuli for MC activation an important group is those signals arising from microorganisms encountered in the human body. These stimuli activate MCs through one of a number of possible pathways discussed below. The first class of receptors for pathogenic material are the Toll-like receptors of which MCs express at least nine [39]. Toll-like receptors are a class of pattern recognition receptors that recognize pathogen-associated molecular patterns (PAMPs) contained in certain molecules produced by pathogenic organisms such as bacteria and viruses. Of the toll-like receptors, TLR2, the receptor for peptidoglycans produced by gram-positive bacteria and fungi [40], seems to be unique in its ability to induce degranulation upon binding of its ligand [41]. TLR4, the receptor for lipopolysaccharide, a molecule produced by gram-negative bacteria, does not induce degranulation upon binding of its ligand. Instead, TLR4 activation leads to the production of a large number of peptide mediators such as interleukins, cytokines and chemokines [39] and the non-peptide arachidonic acid

derivative mediator cysteinyl leukotriene. In addition to the being able to respond to pathogenic microorganisms through the toll-like receptors, another molecule the MC possesses that is capable of recognizing pathogens is the C-Type lectin receptor dectin-1. The major ligands of dectin-1 are molecules containing the β-1,3-glucan motif [42] as found in peptidoglycans from fungi and bacteria. Dectin-1 has a partial ITAM domain in its cytoplasmic domain that while not the complete ITAM is able to bind Syk and induce the expression of TNF-α as well as several other mediators [42]. The CD2 family glycosyl-phosphatidyl-inositol anchored receptor CD48 also contributes to the MCs role in pathogen detection through its ligand, FimH, which is a type 1 fimbrial adhesin expressed by certain bacteria [43]. CD48 is noteworthy in its ability to induce not only degranulation and cytokine production, but also endocytosis of bacterial cells in a caveolar-dependent manner [43]. Pattern recognition receptors are not only expressed on the cell surface, indeed there are multiple pathways for the detection of intercellular pathogens and subsequent activation in response to them. Retinoic acid-inducible gene 1-like receptors (RLRs) and nucleotide oligomerization domain-like receptors (NLRs) are two types of cytoplasmic pattern recognition receptors expressed in MCs. RLRs are DExH/D box helicases that bind to double stranded RNA found in the cytoplasm of cells infected with certain viruses including the viruses that cause dengue, hepatitis C and influenza A [40]. RLRs transduce signals through the caspase recruitment domain (CARD) activation of which ultimately leads to induction of NF-κB activity and augmentation of gene expression [44]. NLRs possess domains that bind microbial motifs [45], an oligomerization domain and a CARD or other similar domain and at least one

NLR, Nod1, was shown to be active in MCs [46]. Pattern recognition receptors are an important group of molecules, the understanding of which may be important for the development of MC related therapies as well as our basic understanding of immune surveillance and response to pathogenic invasion.

In addition to responding to xenobiotics and pathogens, MCs can also be activated by a large number of signals from other cells in the body. As is the case with the pathogen associated pattern recognition receptors, the pathways leading to MC activation by other cells is the product at least in part, of the high diversity of receptors expressed of their cell surface [38]. Not surprisingly given their ubiquity in the biology of all multicellular organisms, several G protein coupled receptors (GPCRs) are expressed by MCs and play roles in the activation of MCs in response to various stimuli. GPCRs are seven transmembrane domain proteins that transduce extracellular signals to the cell through association with GTP-binding proteins on their cytoplasmic tails. Binding of a ligand to the binding pocket of a GPCR leads to the binding of GTP and subsequent activation of adenylyl cyclase, which in turn catalyzes the synthesis of the second messenger molecule, cyclic AMP (cAMP), which triggers a phosphorylation cascade leading to various cellular responses. The majority of ligands for GPCRs expressed on MCs are peptides, either neuroregulatory peptides released by neurons, members of the complement system or toxins produced by pathogenic organisms [40]. One of these classes of GPCRs is relatively newly described and appears to be expressed only on MCs and some nervous cell types found in the dorsal root ganglion. This class is made up of receptors that bear homology to the oncogene Mas1 and are therefore termed the Mas-

related GPCRs (MRGs). There are 7 sub-families of MRGs, however only one of these subfamilies, the X sub-family, found to be present in humans. Within the X sub-family, human MCs are found to express X2 (MRGPRX2). Among the activating ligands of MRGPRX2 are substance P, compound 48/80 and vasoactive intestinal peptide [47]. Another very important group of GPCRs expressed on human MCs are the complement receptors. The complement system is a part of the innate immune system that helps to target and clear pathogenic organisms from the body. Circulating proteins C5 and C3 are proteolytically modified to produce C5a and C3a respectively, which are themselves ligands for GPCRs C5aR and C3aR, present on the surface of MCs [48]. Binding of these peptides to their receptors on MCs leads to histamine release from the MCs which in turn augments the vascular tone of the surrounding vasculature. Similarly to the complement factor receptors MCs also express the GPCR for endothelin which also has a potent effect on local vascular tone, causing vasoconstriction in the lung and blood vessels and also has signaling properties in the cardiac musculature [49]. Binding of endothelin to its receptor on MCs induces degranulation of the MCs. This degranulation then sets up an inhibitory feedback loop due to the release of inactivating peptidases released from MC granules [50].

One of the molecules responsible for activating MCs in response to signals from other cells is the IgG receptor FcγRI [51]. Like FcεRI, FcγRI contains a cytoplasmic ITAM domain which upon binding of an IgG molecule becomes phosphorylated and leads to MC activation. Unlike FcεRI however, there is also an inhibitory receptor, FcγRIIB, which contains an immunoreceptor tyrosine-based inhibitory motif (ITIM)

which upon binding of IgG to the extracellular domain can inhibit activation of MCs [43].

II.3 *In vitro* Growth and Differentiation of Human MCs

II.3.i Isolation of Human MCs

The isolation of MCs from human tissue was first described over 30 years ago [52]–[54]. For example, human skin MCs were isolated from human foreskin removed during circumcisions performed on both newborns and adults. In addition to foreskin, human MCs were also isolated from lung tissue. These early analyses compared the differences between the age of donors and tissue source and began to identify phenotypic differences in MCs derived from different tissues. The protocol developed by these pioneering researchers has stood largely intact to this day. The basic procedure involves mechanically "mincing" the tissue using surgical scissors (**Figure 2.1**) then digesting it with a combination of hyaluronidase, collagenase and DNase in order to disperse cells from the remaining intact tissue. Dispersed cell slurries are then filtered to remove any undigested connective tissue then subjected to centrifugation on a density gradient medium such as Percoll. Density gradient centrifugation is performed in order to remove contaminating red blood cells from the cell slurry. Using this method, the researchers at the time reported a yield ranging from 200,000 to 1,700,000 cells from every gram of tissue used and that these cells were between 3 and 10% pure with respect to MCs (**Table 2.1)**.

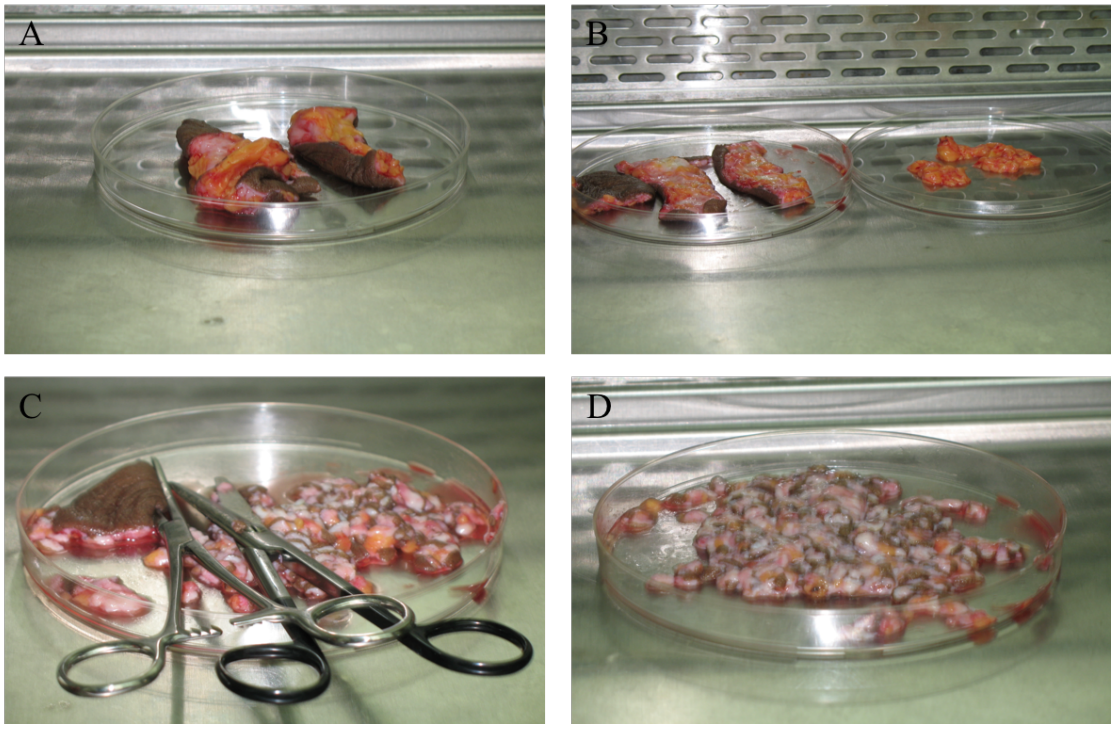

Figure 2.1. Mechanical Homogenization of Skin Tissue for MC Extraction. Skin tissue is shown in various states of mechanical homogenization by surgical scissors and hemostats. Excised tissue (A) is first cleared of subcutaneous adipose tissue (B), then cut into cubes (C-D). All procedures are performed in a biological safety cabinet as depicted.

Table 2.1. Characteristics of Human MC Isolation Protocols. Several MC isolation procedures are reviewed, yields are presented in cells per gram and purity in percentage. Table was created using data collected from references given.

Study	*Tissue Source*	*Cells per gram of tissue*	*Purity %*	*Reference*
Schwartz et al	Foreskin (A/N) Lung	200,000 -500,000 200,000-500,000	6.7/3.2 5.1	47
Lawrence et al	Foreskin (N) Skin (A)	600,000-1,000,000 450,000-550,000	10 10	45
Benyon et al	Foreskin	1,500,000-1,700,000	5.4	46

The cells isolated using this method were investigated for their ability to release histamine in response to various stimuli such as anti-IgE, the calcium ionophore A23187, compound 48/80, poly-L lysine, morphine, substance P [53], f-MLP peptide and C5a [52]. The levels of tryptase and chymase activity in freshly isolated MCs was measured using cell extracts and chromogenic substrates [54]. Given the comparative ease of obtaining healthy skin from human subjects relative to lung, skin MCs are the most well characterized of the primary human MCs. This is discrepancy of ease with which source tissue can be obtained is an important one due to the highly heterogeneous nature of MCs from different tissues. Great care must be taken not to conflate results obtained with one tissue-specific MC type with characteristics common to all MC types.

These foundational studies laid the groundwork for much of the work that was done using MCs isolated from human tissue in their mature form, indeed these three papers have been cited over 700 times in the three decades since their initial publication. Indispensable as these cells and the methods for their isolation have been to the MC

research community, this protocol is not without some considerable drawbacks that must be taken into account. The relatively low numbers of mature MCs present in normal human tissue is the first problem. Mature MCs have an extremely limited proliferative capacity in the human body and in culture [55] and therefore must be expanded once in culture or very large amounts of initial tissue must be used, which can be prohibitive given that the amount of tissue that is obtained is determined by the needs of the donor and thus cannot be dictated by the researcher. The problem of the low numbers of mature MCs in tissue is compounded by the relative inefficiency of the isolation method. Although this lack of efficiency is difficult to quantify and exact data supporting this assertion is lacking, it is the opinion of many practitioners of this protocol that many MCs are lost at multiple steps during the isolation procedure (Personal Observation). Another limitation of this protocol is necessity of the MC cultures obtained to be further purified by long-term culture in serum free media to allow other cell types to die off.

II.3.ii *In vitro* Differentiation of MC

In order to attempt to address some of the technical and biological limitation associated with the isolation of fully mature MCs from tissues and also to further the knowledge of the developmental biology of the MCs, protocols were also developed for the isolation of MC progenitors and the subsequent *in vitro* differentiation of these cells into mature MCs. MC progenitor cells can be obtained from various sources including but not limited to $CD34^+$ peripheral blood [18], [56]–[58], blood taken from the umbilical cord [59], [60], bone marrow [9][54], dispersed fetal liver [62] [63], or embryonic stem

cells [64][65]. The first reports of MCs produced by differentiation of progenitors isolated from peripheral blood came from around the same period as those reporting the isolation of mature MCs from tissues. Initially, differentiation was achieved through co-culture of cells isolated from peripheral blood on so-called "feeder" cell monolayers of NIH-3T3 mouse embryonic fibroblasts [18]. The progenitor cells used in this study were obtained by isolating $CD34^+$ cells from peripheral blood using immune-affinity purification using an anti-CD34 antibody. After purification, MC differentiation was achieved using IL-3 and SCF. This protocol has since been improved upon by the addition of different combinations of differentiating factors, however the basic technique has gone largely unchanged. An alternative source of blood for the isolation of MC progenitors is blood taken from the umbilical cord of mothers gestating or recently birthed newborns [66]. This protocol was also developed around thirty years ago and is still in use today [67]. While obvious limitations exist in terms of obtaining cord blood, it is nonetheless an interesting source of MC progenitors as it affords the possibility of banking cells from the child in question for use in a possible autologous therapy later in life [68]. Much like the protocols for differentiating MCs from peripheral blood, the first studies relied on NIH-3T3 cells as the source differentiating factors, while subsequently developed protocols used recombinant proteins added to the growth media [46]. MC progenitors can also be isolated from the bone marrow as it is the location of the primary phase of MC development as it is indeed also the site of the beginning of all hematopoiesis. Bone marrow in humans is most commonly collected from the iliac crest

by means of a biopsy. The utility of fetal liver MCs outside of basic research settings is limited due to the nature of obtaining healthy human fetal tissue.

Table 2.2 Differentiation of Human Progenitors into MCs. At least five sources have been reported for MC progenitors in humans. The regimens used for differentiation are reviewed in brief.

Progenitor Source	*Progenitor Cell*	*Morphogens*	*Duration*	*Reference*
Peripheral Blood	$CD34^+$ PBMC	SCF, IL-3	21 days	[69]
Umbilical Cord Blood	$CD34^+$ cord blood cell	SCF, Flt-3 ligand, thrombopoietin	4-16 weeks	[70]
Bone Marrow	$CD34^+$ bone marrow cell	SCF, IL-6	12 weeks	[71]
Fetal Liver	Unknown	SCF	35 days	[63]
Embryonic Stem Cells	$CD34^+CD43^+$ cell	SCF, IL-3, IL-6, Flt-3 ligand	10-14 weeks	[65]

While the exact regimen used to differentiate these cells into MCs depends on the study and are reviewed in **Table 2.2**, most make use of recombinant cytokines such as IL-3, IL-6 and SCF. Differentiation protocols generally take multiple months to complete and often require several different differentiating molecules administered at intervals. These protocols can allow production of MCs in numbers sufficient for laboratory studies. It is unclear to what extent these cells recapitulate the phenotype of either skin or mucosal MCs, however it seems clear that they are preferable to the use of rodent MCs or transformed human MC lines.

II.4 Human Mast Cell Lines

II.4.i HMC1/2

The human MC (HMC-1) line was created in 1988 at the Mayo Clinic from cells taken from the peripheral blood of a patient with "mast cell leukemia" [72] and constitutes the first human MC line. Since its creation, the HMC-1 cell line has contributed to countless discoveries, with the initial paper describing it bearing well over five hundred citations. These MCs were found to be most similar to immature MCs in that they did not contain significant levels of histamine nor did they express FcεRI, however they did express the receptor for stem cell factor c-KIT, albeit a mutated version of it. It was determined using sequencing and site directed mutagenesis that HMC-1 carried an allele of the *c-kit* gene that carried a missense mutation that changed an aspartic acid residue to a valine. This substitution in turn created a constitutively active receptor that lead to the hyperplasia observed in the original donor and conveyed the HMC-1 cell line to continue to proliferate in culture [73]. These cells were found to express the major histocompatibility complex class II antigen molecules HLA-DR, HLA-DQ and HLA-DP, of which HLA-DR and HLA-DQ were found to be inducible by recombinant human interferon gamma (rhIFN-γ) and recombinant human IL-4 (rhIL-4) [74]. Further investigation revealed that HMC-1 expressed functional β-tryptase as opposed to α-tryptase, and interestingly, while HMC-1 lacked the alpha and beta chains of the FcεR, they showed strong expression of the gamma chain (which is shared by the Fcγ receptor) [75]. One of the greatest utilities of the HMC-1 cell line was and indeed continues to be, in the study of MC leukemia, or what we now refer to as mastocytosis,

which is very often the result of similar activating mutations in *c-kit*. However as useful as it may have been, it also has many obvious drawbacks including its lack of FcεRI expression, lack of histamine, mutant form of *c-kit* and inability to degranulate in response to appropriate stimuli.

II.4.ii LAD2

Citing the problems mentioned above with the HMC-1 human MC line, researchers at the Laboratory of Allergic Diseases (LAD) at the NIH in 2002 established and characterized a second human MC line LAD2 [76]. These cells were taken from the bone marrow of a patient also suffering from mastocytosis. From this donor, five cell lines were reportedly created, LAD 1-5, however only LAD1 and LAD2 were reported on in the initial publication of these findings and of those, LAD2 is the most widely used today. Cells grown from the initial bone marrow sample were cultured for 24 months before being designated as official cell lines. Following this period of culture, the cells were cryogenically stored and reconstituted, after which point various phenotypic and functional tests were performed to determine the degree to which these cells resembled mature MC isolated from human tissue. LAD cells were found to be roughly 98% tryptase positive and 37% tryptase/chymase positive and contain between three and six times as much histamine as HMC-1. Degranulation could be induced by IgE mediated FcεRI aggregation and FcγR upregulation could be achieved using IFN-γ. Importantly, unlike HMC-1 cells, LAD cells were found to possess the wild type allele of *c-kit* and thus were dependent on the addition of SCF to their growth media in order to proliferate.

While LAD2 cells do not carry the activating mutation in *c-kit* and are dependent on SCF, they are transformed, leukemic cells and therefore cannot be considered normal human MCs. In the years since the establishment of the LAD2 cell line it has been described as having exceedingly low levels of tryptase and chymase [12][77] as well as unacceptably large variability in terms of basic characteristics like doubling time and response to cryopreservation [78]. While there are significant limitations concerning the use of LAD2 cells, the creators of this cell line continue to tout their utility [79], citing a somewhat steady rate of materials transfer agreements and licensing agreements being entered into in the years since their establishment.

II.4.iii LUVA

The final human MC line was developed at the University of Virginia in 2011 and are called LUVA (named after Dr. Larry Borish at UVA who first described them). They were reported to arise from a population of $CD34^+$ cells taken from the peripheral blood of a patient who appeared healthy with respect to mastocytosis but was suffering from aspirin-exacerbated respiratory disease. Similar to LAD2 cells, LUVA cells did not carry a mutated form of *c-kit*, however in stark contrast to LAD2 cells could be cultured in the absence of SCF. This result in combination with the lack of any detectable mastocytosis in the original donor, remains perplexing and unexplained and is worthy of further investigation. Phenotypically LUVA cells were found to express c-KIT, FcεRI, FcγRII, tryptase and chymase. They were reported to degranulate in response to IgE-mediated FcεRI aggregation as determined by release of β-hexosaminidase, thromboxane A_2 and

prostaglandin D_2 [78]. In the seven years since they were first described, a modest amount of work has been done with LUVA cells and they may well constitute the closest approximation of any currently available immortalized cell line of a normal human MC. One major impediment to their use is the aforementioned question of the mechanism of their immortalization that will need to be resolved.

II.5 Animal Models of MCs

II.5.i Mouse Models

The first mouse model used in the study of MC biology was a line that carried a mutation in the *white* locus. These mouse among several other hematopoietic deficiencies, showed an almost complete lack of MCs [80]. The white locus has since been found to be the location of the gene for the stem cell factor receptor c-KIT. The W/W^{-v} mouse line, as it was named, carried two copies of a non-functional *c-kit*. Seminal bone marrow transplant experiments were performed with these mice demonstrating the bone marrow origin of the MC precursor. This mouse model has been a workhorse of the MC biology field; however, its utility may be approaching its end as we learn more about it. Disregarding for now the significant differences between murine and human MC biology, the W/W^{-v} mouse model is problematic because the MC development is far from the only process controlled by c-kit. Indeed c-kit plays a role in many hematopoietic processes, and therefore attributing observations seen in these mice solely to the lack of MCs simply cannot be done [81].

II.5.ii Caveats with Murine Mast Cell Biology

The most well characterized animal models of MCs come from rodents. This is peculiar in a sense as rodent and human MC biology is very divergent, indeed much more so than would be expected given the close phylogenetic positions of humans and rodents and certainly more than would be expected given the power rodent models have had in the past when providing models for human biology. There are many reasons for this including the fact that human IgE does not bind to mouse FcεRI, the fact that MCs in rodents arise from both the bone marrow and spleen, whereas MC in humans are thought to arise only from the bone marrow.

II.6 Adipose Derived Stem Cells (ADSCs)

The initial description of adipose derived stem cells (ADSCs) was in 2001 in a landmark paper that has since been cited over 4,500 times [82]. The motivation of this work was to provide an accessible source of autologous adult stem cells. At the time this work was done the most common source of adult stem cells was bone marrow mesenchyme [83]. Bone marrow contains two types of stem cells, hematopoietic stem cells that give rise to all the cells in the blood and mesenchymal stem cells which are found in the mesenchymal stroma and which give rise to bone, fat, cartilaginous [84] and muscle tissue producing cells [85]. While useful to the field of tissue engineering, the highly invasive nature of bone marrow collection and the low numbers of cells obtained in this way drove the authors to search for an alternative. It was understood at the time, based on studies in animals of various vertebrate groups including rodents [86], birds [87]

and rabbits [82], that connective tissue found in and around organs contained mesenchymal stem cells, similar in differentiability to the mesenchymal stem cells obtained from bone marrow. Adipose tissue was chosen as a potential source of mesenchymal stem cells as contains a significant amount of connective tissue, but more importantly because adipose tissue, like the bone marrow is formed from the embryonic mesoderm and therefore it was postulated that stem cells derived from it should have similar lineage commitments [88].

II.7 Stromal Vascular Fraction (SVF)

Adipose tissue is a complex mixture of cells, but can be separated experimentally into two fractions, adipocytes in one and all remaining cells in another. The non-adipocyte fraction is termed the Stromal-vascular fraction (SVF) and has been recognized and utilized since 1964 when collagenase digestion was used to liberate adipocytes from rat adipose tissue [89]. Since its establishment in 1964, the SVF has been used in the study of adipocyte biology [90]–[92].

II.8 ADSC Isolation

When the isolation of human ADSCs was first performed, SVF cells were isolated from human liposuction aspirate by digestion with 0.075% collagenase followed by centrifugal fractioning. SVF cells were plated and allowed to adhere overnight, the cells remaining after a media change to remove non-adherent cells were termed processed lipoaspirate (PLA) cells. PLA cells were cultured in DMEM supplemented with 10%

FBS, 1% Antibiotic/Antimycotic. PLA cells were subjected to multiple forms of interrogation including flow cytometry, immunofluorescent staining, determination of doubling time and senescence, and differentiation capacity [82]. While various minor changes have been made to this protocol, much of the process has remained unchanged. Some of the improvements and alternative methods are discussed in more detail.

II.8.i Source of Adipose Tissue

The composition of adipose tissue is dependent on which part of the human body it is collected from and the properties of the cells collected depend on the manner in which it was removed. The two forms of human adipose tissue that are commonly available to researchers are bulk adipose tissue removed during surgery and the aspirate collected during liposuction procedures. While surgically removed adipose tissue may come from many parts of the body, it is generally subcutaneous adipose tissue collected from the breast, abdomen or lower extremities (Personal Observation). Omental adipose tissue may also be used as a source of ADSCs [93], however due to the invasive nature of its collection, is not an ideal candidate as a source of autologous stem cells for therapies. Liposuction aspirate is almost exclusively abdominal (Personal Observation) and it has been shown that stem cells can be isolated from one of two fractions [94], that are analogous to polar and non-polar phases in a mixture of two immiscible liquids. A comparison was performed on of the characteristics of ADSCs taken from the abdomen or the waist (hip or thigh) [95]. This study demonstrated that the differentiative capacity of cells taken from these two sites were similar, with no statistically significant difference

observed in osteogenic or chondrogenic differentiative capacity. A difference was observed in the number of differentiatable cells, with the abdominal tissue yielding roughly five times as many cells as tissue taken from the waist. In a study of ADSCs in mice it was found that significant differences were observed in the characteristics of stem cells derived from fat pads in different organs [96], classed in the study as either visceral or subcutaneous adipose tissue. Another comparative study was performed to determine the effect of adipose tissue collection method on the characteristics of the stem cells yielded [97]. In this study, cells derived from either bulk tissue or liposuction aspirate collected using a power assisted or laser assisted technique were investigated for their proliferative and differentiable qualities, with the laser assisted liposuction yielding the lowest quality stem cells.

II.8.ii Isolation Technique

In addition to differences stemming from the location of the adipose tissue collected, at least three different techniques for isolation have been developed. The first alternative method is the explant or bulk tissue culture method, which is performed by simply placing adipose tissue in cell culture media and allowing the ADSCs to exfiltrate the tissue [98][99]. While these methods are attractive given the ease and low number of consumables used, these methods are significantly less efficient and are likely to yield a population of cells enriched in motile cells that may or may not be useful for subsequent therapies. The second alternative method for the isolation of ADSCs is the use of enzyme-free, mechanical isolation protocols designed with human therapies in mind that

make use of hydrodynamic shear forces to disrupt intact tissue within liposuction aspirate [100]. These procedures yield what is known as “Nanofat” and is performed by serially passing liposuction aspirate back and forth between two luer-locked syringes. This method was developed and popularized by cosmetic surgeons for whom the use of emulsified adipose tissue is beneficial for various processes in cosmetic surgery [101]–[103]. This technique is attractive for the same reasons as the explant culture method, however its success depends on the nature of the tissue being used, with bulk tissue being unsuitable and certain liposuction aspirates also being unsuitable depending on the bore of the canula used during the liposuction procedure (Personal Observation). The third type of isolation techniques makes use membranes to separate the ADSCs from the other cell types found in the SVF. The simplest of the membrane-based techniques is the membrane filtration technique [104], in which the enzymatically dispersed SVF is passed through a single membrane of polyurethane with an average pore size of 11 μm. Due to the low purity of the cultures obtained in this way, the membrane filtration procedure was then further modified to include an intermediate incubation step wherein the membrane used for filtration is incubated in a tissue culture dish to allow cells bound to the membrane to detach and migrate onto the surface of the plate [105]. This additional step reportedly improved both the extent of mesenchymal stem cell markers as well as the ability of these cells to differentiate into osteoblasts [106].

II.9 Differentiative Capacity of ADSCs

As previously discussed, adipose tissue is derived from the embryonic mesoderm and ADSCs are most commonly differentiated into muscle, bone, fat and cartilage as summarized in **Table 2.3**, although the differentiation of these cells into other cell types has also been reported, including cardiomyocytes [107], neurons [108] and endothelial cells [109] as summarized in **Table 2.4**.

Table 2.3. Differentiation Protocols for Adipogenic, Chondrogenic, Osteogenic and Myogenic Differentiation of ADSCs. Summarized are the four most common cell types produced by differentiation of ADSCs.

Cell Type	*Morphogen*	*Duration*	*Reference*
Adipocyte	Isobutyl-methylxanthine, dexamethasone, insulin, indomethacin	2 weeks	[82]
Chondrocyte	Insulin, TGF-β1, ascorbate-2-phosphate	2 weeks	[82]
Osteoblast	Dexamethasone, ascorbate-2-phosphate, *β*-glycerophosphate	2 weeks	[82]
Myocyte	Dexamethasone, hydrocortisone	6 weeks	[82]

Table 2.4. Differentiation Protocols for Cardiomyogenic, Neurogenic, Epithelial and Endothelial Cell Differentiation of ADSCs.

Cell Type	*Morphogen*	*Duration*	*Yield*	*Purity*	*Reference*
Epithelial Cell	All-*trans* retinoic acid	10 days	NA	~80%	[110]
Endothelial Cell	Hydrocortisone, vitamin c, heparin, EGF, FGF2, IGF-1, VEGF	6-10 days	NA	NA	[111]
Neuron	Butylated hydroxyanisole, KCl, valproic acid, forskolin, hydrocortisone, insulin	>24 Hours	NA	NA	[112]
Cardiomyocyte	5-azacytidine or trichostatin A	3 weeks	NA	NA	[113]

II.10 ADSC Phenotype

II.10.i Morphology

Morphologically, ADSCs in culture appear indistinguishable from fibroblasts. They are characterized by their long, spindle-shaped appearance and their propensity to adhere to tissue culture plastic. ADSCs grown in culture grow and divide rapidly and exhibit contact inhibition. Considerable variation is observed in proliferation and heterogeneity of morphology in cell preparations produced from different donors (Personal Observation).

II.10.ii Immunophenotype

Considerable debate exists in the field of ADSC biology about the exact immunophenotype of the ADSC. This discrepancy is almost certainly the product of the varied protocols used for isolation of ADSCs as well as the highly heterogeneous nature of the SVF. While certain CD markers are widely agreed upon throughout the field as either positive or negative markers of ADSCs, others appear as positive markers in some studies and negative markers in others. Resolution of these disputes will be necessary as human therapies are designed using ADSCs. A meta-analysis of 16 publications from 2002 to 2016 ([94], [114]–[128]) showed that out of 48 cell surface markers, 15 were unanimously reported as positive (**Table 2.5**), 18 negative (**Table 2.6**) and 15 were reported as both positive and negative markers in two or more reports (**Table 2.7**).

Table 2.5. Undisputed Positive ADSC Markers. Out of sixteen publications describing positive markers of ADSCs, all agree upon the identity of these markers as positive.

Undisputed Positive Markers	*Number of Reports*
CD9	4
CD10	3
CD13	4
CD29	6
CD36	1
CD44	7
CD49e	2
CD51	1
CD55	4
CD59	4
CD90	15
CD117	2
CD133	1
CD142	1
HLA-ABC	3

Table 2.6. Undisputed Negative ADSC Markers. Out of sixteen publications describing negative markers of ADSCs, all agree upon the identity of these markers as negative.

Undisputed Negative Markers	*Number of Reports*
CD3	1
CD11	1
CD11a	1
CD11b	3
CD11c	1
CD14	6
CD16	3
CD18	3
CD19	1
CD20	1
CD31	8
CD35	1
CD45	13
CD50	3
CD56	4
CD62	2
CD73b	1
CD104	3

Table 2.7. Disputed ADSC Markers. Out of sixteen publications describing either positive or negative markers of ADSCs, the CD molecules in this table appeared at least once as both positive and negative markers of ADSC identity.

Debated Markers	*Number of Reports Positive*	*Number of Reports Negative*
CD34	9	7
CD49b	1	1
CD49d	5	1
CD54	4	1
CD61	1	2
CD62e	1	2
CD63	1	1
CD71	4	1
CD73	9	1
CD105	13	2
CD106	3	4
CD140a	1	1
CD146	4	3
CD166	4	1
HLA-DR	1	4

II.11 Breast Cancer

Breast cancer is the most common form of cancer in women regardless of age, race or ethnicity. Furthermore, with the exception of prostate cancer in certain ethnic groups, breast was the most common site of all cancers in the United States between 2010 and 2014. According to the Centers for Disease Control and Prevention statistics, roughly 236,000 women were diagnosed with breast cancer in 2014, and roughly 41,000 women lost their lives as a result of having the disease. According to the same study, the rate of

incidence among women of all races was 123.9 per 100,000 women, higher than for any other site in the body [129].

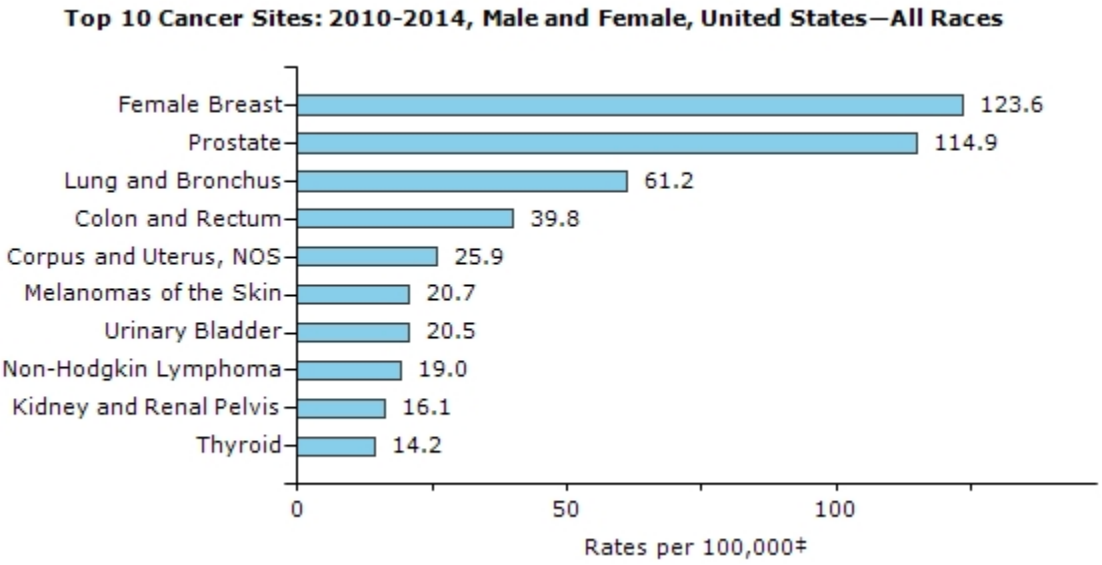

Figure 2.2. Rates of Cancer Among Americans Between 2010 and 2014. Centers for Disease Control and Prevention statistics showing the ten most common locations of cancer among Americans over a four year period showing breast to have the highest prevalence [129]**.**

Breast cancer is a highly heterogeneous disease which exhibits varying degrees of severity, morphology, mutational burden and mechanism of progression. One method of classification of breast cancer is through the expression of certain compliments of genes [130]. Using this system of classification breast cancers can be first grouped by their expression of the estrogen receptor (ER) and thereafter by the duplication [131] and overexpression of the oncogene *Erb-B2,* which was found to be expressed in up to 30% of all breast cancers [132]. While this system of classification was useful, it has since been improved upon, in particular with the recognition of the role played by the

progesterone receptor (PR). Much of the early work and many of the early therapies targeted one or more of these three proteins with a high degree of success. In at least 12% of female breast cancer patients the expression of all three of these genes are ablated and their tumors are therefore refractory to steroid hormone treatment and any therapy targeting the *Erb-B2* gene product, HER2, these tumors are termed triple negative [133].

II.11.i HER2 in Breast Cancer

HER2 is a member of the epidermal growth factor receptor (also known as ERBB [134] and HER) family [135], which are transmembrane tyrosine kinase receptors. This family is composed of four members, namely HER2-4 (also known as ERBB2-4) and the epidermal growth factor receptor or EGFR (also known as ERBB1). As with other receptor tyrosine kinase proteins, binding of the cognate ligand to the extracellular domain induces dimerization with other ERBB receptors forming either homodimers or heterodimers. Ligand binding and dimerization induce conformational changes and interactions that lead to phosphorylation of tyrosine residues within the cytoplasmic domains of the two receptors. The phosphorylated tyrosine residues in the cytoplasmic domains create binding sites for other cytoplasmic signaling molecules that are able to activate or inhibit signaling pathways in a variety of ways. Activated ERBB family receptors predominantly signal through the mitogen-activated protein kinase (MAPK) pathway or the phosphatidylinositol 3 kinase (PI3K) pathway, however they also interact with and signal through members of the STAT family, the tyrosine kinase SCR and the master regulator mammalian target of rapamycin (mTOR).

Ligands for these receptors include epidermal growth factor family ligands summarized in **Table 2.8** [136].

Table 2.8. Ligands for the Four ERBB Family Receptors. Ligands for the ERBB family receptors are grouped according to their shared or unique binding to different isoforms [136]**.**

Receptor	*Specific Ligands*	*Shared Ligands*
EGFR	EGF, transforming growth factor α, amphilregulin	Betacellulin, heparin binding EGF, epiregulin
HER2	None	None
HER3	None	Neuregulin 1, Neuregulin 2
HER4	Neuregulin 3, neuregulin 4	Betacellulin, heparin binding EGF, epiregulin, Neuregulin 1, Neuregulin 2

Although HER2 does not have a soluble cognate ligand, it does have a membrane associated ligand MUC4, the binding of which leads to the phosphorylation of at least one tyrosine residue, Tyr-1248, which is creates a binding site for the protein downstream of kinase related (DOK-R) [136] and may play a role in receptor localization of HER2 [137]. Despite the fact that HER2 does not bind any of the ligands bound by other ERBB family members, it nevertheless plays an important role in modulating the activity of other ERBB family receptors through ligand induced dimeric interactions with other receptors. Indeed, HER2 has been shown to be the preferred heterodimeric binding partner of ligand-bound ERBB receptors [138].

In the context of cancer biology, ERBB family receptors play an important role, indeed EGFR has been recognized for its role in the progression of cancer for over three

decades [139]. ERBB family receptor activity is typically found to be irregularly high in cancer cells, with this increase in activity arising by various mechanisms. In certain instances, the number of receptors is increased due to increases in gene copy number, with duplications of the genes important events of the progression of cancer development. The most commonly duplicated genes in the *Erb-b* family are EGFR [140] and HER2 [141]. In other instances, where the gene copy number and receptor number are not increased, paracrine and autocrine signaling stimulate receptor activity [142]. Activation of ERBB family receptors through any of the aforementioned mechanisms lead to increased cell proliferation as well as production of angiogenic signals such as vascular endothelial growth factor (VEGF) [143].

II.11.ii The Role of MC in Cancer

MCs are most commonly associated with their role in Type I hypersensitivity [144], however given their ubiquitous distribution in tissue, their role in all forms of cancer has been studied extensively [3][145][146]. MC are the first immune cells to localize to tumors and to enter the tumor microenvironment, yet their contribution to tumor progression and metastasis is highly tumor-specific and widely debated [147]. It has, for example been shown in some animal models that MC can have pro-tumorigenic effects, while in other models or even in some cases in the same tumor, MC seem to have an anti-tumorigenic effect. These seemingly conflicting results may be the result of differences in the subtype, stage, or grade of the tumors investigated or the various methods used to identify MCs. Furthermore, differences in rodent and human MC

biology are many and therefore the results obtained using these models do not accurately reflect the corresponding human disease state and can ultimately lead to misleading conclusions [148], [149][150]. There is clearly a great deal that remains unknown about the role of MC in cancer and many important questions remain unanswered.

Despite these outstanding questions, it is also clear that in at least certain human cancers, namely breast and colo-rectal cancers, the presence of MC in particular parts of the tumor can be associated with a favorable survival outcome [8][151][152][4]. As previously discussed, one of the components of MC granules is TNF-α, indeed they are one of the only cell types in humans that have pre-formed TNF-α that can be released upon activation of FcεRI [153][154]. In addition to TNF-α, MC can synthesize and release large amounts of granulocyte-macrophage colony-stimulating factor (GM-CSF), up to 4 ng/mL from 100,000 cells [155][156], reactive oxygen species, prostaglandin D2, IL-9 and heparin [3], many of which have potential anti-tumorigenic effects. The effect of the various molecules released from MC in the breast cancer tumor microenvironment remains to be determined.

II.11.iii The MC Cytokines TNF-α and GM-CSF as Anti-Cancer Agents

Tumor necrosis factor α was first described in the 1970s [156][157] and has since been shown to be a cytokine that is highly multifunctional and consequently it plays roles in multiple parts of human physiology, such as regulating vascular tone through its interaction with vascular endothelial and smooth muscle cells. TNF-α also, as its name might suggest, has the ability to inhibit tumor cell proliferation and in certain cases

promote tumor regression. TNF-α can also function in the form of an adjuvant, increasing the efficacy of chemotherapeutic drugs such as doxorubicin or a EGFR tyrosine kinase inhibitor [158]. The most significant drawback when considering TNF-α as an anti-cancer drug is its toxicity when administered systemically, leading to the investigation of its utility in the treatment of tumors in which local administration is contained by the surrounding organ. One method of administering TNF-α that has shown some success has been through isolated limb perfusion of TNF-α [159] in combination with drugs such as melphalan [160][161], or doxorubicin [162] introduced under hyperthermic conditions [163].

GM-CSF is another cytokine produced by MC that is being examined for its potential utility as either a treatment [164], or an addition to immunotherapeutic approaches to treating breast cancer such as trastuzumab IgG [165] or docetaxel [166]. One such approach uses the immunogenic peptide E75 in conjunction with GM-CSF to treat tumors in breast cancer patients. Using this approach, there was an observed reduction of 48% in the relative risk of recurrence when E75 was administered with GM-CSF as compared to E75 alone, a finding that paved the way for a Phase III clinical trial of this combination [167]. In another study of individuals with metastatic trastuzumab-resistant HER2 positive breast cancer, GM-CSF co-administration with trastuzumab was shown to have increased therapeutic effect as compared to trastuzumab alone [165]. In addition to these studies, more than 50 clinical trials are either complete or in progress which examine the potential benefits of GM-CSF in cancer treatment, providing encouraging evidence for its use in cancer therapies.

II.12 Allergo-Oncology

The association between allergy and cancer has been the topic of investigation since at least 1955, when a study was done to examine the effect of an allergic inflammatory response to tumor grafts in mice [168]. This investigation did not show any effect of allergic response on tumor progression, however research continued into possible connections between allergy and cancer. In 1976 an association study was done to compare the prevalence of atopy in cancer patients which reported that atopy was found at rate fifteen folds less than in non-cancer bearing individuals [169]. In the 1980's studies were done that showed that histamine levels in patients with tumors were decreased compared to a control group and that upon removal of the tumor load, the patients histamine levels returned to normal levels [170]. Similar results were obtained when response to histamine was measured in the skin of cancer-bearing versus non-cancer bearing individuals, where patients with tumors or having recently had tumors removed showed a diminished ability to respond to sub-dermal injections of histamine [171]. Initially, the decreased level of histamine in cancer patients was attributed to the observation that these patients had lower numbers of circulating basophils in their blood as compared to non-cancer bearing control patients [172]. Basophils isolated from the tumor bearing population were found to be normal with regards to histamine content and ability to degranulate. It is interesting to note that MCs were not examined in this study, however it may not have been feasible to obtain MCs from patients to perform the study. In 2005 a study of over one million participants was performed to determine the association between risk of mortality from cancer and hay fever/asthma which found a

decreased risk of mortality from cancer in individuals who reported that they had either hay fever or asthma [173]. Given these reported associations between cancer and allergy and given the growing desire to engage the immune response in destroying tumors in cancer patients, the question of whether the allergic response could be used in destroying tumors began to be asked [174] and the field of allergo-oncology was born. The term "allergo-oncology" seems to have originated or at least became popularized around the year 2008 [175] and refers to the use of the allergic hypersensitivity reaction to fight cancer. Of particular interest to the nascent field of allegro-oncology was the IgE molecule, the immunoglobin most commonly associated with hypersensitivity and the allergic arm of the immune system in general. The IgE molecule and its receptor have many attractive properties from the point of view of therapy development that in some cases may make it a more sensible platform than IgG. The first and perhaps most obvious advantage of IgE is its affinity for its receptor. The interaction of IgE with the FcεRI receptor is orders of magnitude higher than that of IgG for its receptor. This very high affinity of IgE for its receptor means that upon receptor binding, IgE molecules tend to stay bound to their receptors and are therefore able to decorate effector cells for longer periods, reportedly for up to several months, or certainly for the lifetime of the cell to which they are bound [175]. In part due to the high affinity for the FcεRI receptor and its resultant sequestration, IgE levels in the blood tend to be kept very low [176]. In mice it was shown that IgG subtypes had serum half-lives of between 4 and 8 days, whereas IgE had a half-life of only 12 hours [177]. While these timescales are likely to be slightly different in humans, they are nonetheless a good illustration of the stark difference in

serum persistence among the different immunoglobin classes. The advantage of the short half-life of IgE is that it minimizes the risk of exogenously administered antibodies to induce systemic anaphylaxis, a major possible complication for any antibody-based therapy. In addition to its short half-life, the concentration of IgE is also kept very low, with IgE making up only 0.02% of the total complement of Ig's in the blood, IgG on the other hand, accounts for up to 85% of the total Ig complement [175]. This may serve to create less competition for free receptors for IgE as compared to IgG where the majority of receptor sites are likely to be occupied.

II.12.i MOv18

One of the first reported uses of allergo-oncology came in 1999 [178] when an IgE recognizing the folate receptor was created and assessed for its ability to kill ovarian cancer cells. In this study, MOv18-IgG1, a previously characterized IgG1 specific to the folate receptor, was used. MOv18-IgG1 had been shown previously to be capable of inducing antibody-dependent cell-mediated cytotoxicity (ADCC) of an ovarian cancer cell line by peripheral blood mononuclear cells [179]. In order to start to understand the effect of Ig subtype, a chimeric antibody was created that fused the Fc domain of IgE to the variable domain of MOv18-IgG1. The resultant antibody was compared to the original IgG1 for its ability to induce ADCC.

The choice to target the folate receptor and ovarian cells was based several factors including the previous observation that ovarian cancer was less prevalent in individuals with high levels of allergic activity [173], the high degree of fatality associated with this

disease and the existence of previously validated models of IgG1-mediated tumor cell cytotoxicity, namely the cell line IGROV1 and the MOv18-IgG1 antibody. It was determined in a direct comparison between the two antibodies, MOv18-IgE and MOv18-IgG1 that the IgE-based antibody induced significantly more tumor cytotoxicity than did the IgG1-based antibody and did so for a longer duration in a severe combined immune-deficient (SCID) mouse xenograft model of ovarian cancer. The same group of researchers followed this study with an investigation of the same MOv18-IgE in a nude mouse model [180]. In this study, human ovarian cell tumor tumors were induced in nude mouse in conjunction with human peripheral blood-derived monocytes. Using this model system investigation of the contribution of IgE-mediated monocytic tumor cytotoxicity was possible due to the fact that human IgE does not bind mouse FcεRI. The authors reported that MOv18-IgE was able to induce ADCC both *in vivo and in vitro.* In the *in vivo* experiments, where injection of human monocytes and MOv18-IgE resulted in tumor infiltration by monocytes and was able to increase survival in tumor bearing mice to a higher degree than MOv18-IgG1. In the *in vitro* studies, MOv18-IgE was able to induce ADCC and facilitated phagocytosis of cancer cells by IgE-bearing monocytes, a process termed antibody-dependent phagocytosis (ADCP). Following on from this work, an investigation into the mechanism of IgE-mediated ADCC was undertaken [181], namely the differentiation between ADCC and ADCP and to determine to what extent the two FcεR's are involved in these processes. Using a sophisticated, three color flow cytometry assay, the authors were able to show that the high affinity IgE receptor FcεRI

mediated ADCC of cancer cells by human monocytes, whereas the low affinity IgE receptor CD23 meditated ADCP.

II.12.ii HER2

After the success of MOv18 and the targeting of the folate receptor to induce cancer cell targeting and killing, other targets were investigated, perhaps the most promising of which was the protein HER2. HER2 was chosen in part due to the existence of an IgG molecule targeting HER2, trastuzumab and the success it had shown as the antibody-based therapy Herceptin. The first development of IgE molecules targeting HER2 was achieved using an oral-administration based vaccination of mice with peptides engineered to mimic the epitope of HER2, or "mimotopes", bound by trastuzumab [182][183]. Using this strategy, HER2 antibodies produced by mice were able to induce tumor cell killing when incubated with a rat basophil cell line. In light of these successes with targeting HER2 on cancer cells and the success of the work performed to create and employ a humanized IgE-IgG chimeric antibody, Trastuzumab-IgE was created and characterized [184]. The resultant antibody was found to be capable of ADCC and was able to induce degranulation of MCs when incubated with HER2 positive cancer cells. A comparison of trastuzumab-IgE showed comparable cytotoxic effects to trastuzumab, however the trastuzumab-IgE mediated ADCC, whereas Trastuzumab mediated ADCP. The trastuzumab-IgE antibody is a humanized antibody, but is not fully human, for this reason a fully human IgE targeting HER2 was created [185]. The antibody, designated as C6MH3-B1, was able to induce degranulation of

basophils when co-incubated with HER2 positive cancer cells, similar to the previously examined antibodies. C6MH3-B1 was also shown to play a role in antigen presentation by human dendritic cells *in vitro*. The authors also investigated the ability of the soluble, extra-cellular domain of HER2 (ECD^{HER2}) to induce degranulation of basophils decorated with C6MH3-B1, which it did not. This was an important piece of information as HER2 is known to shed part of its extracellular domain into a soluble form that can be detected in the bloodstream. In the event that soluble ECD^{HER2} was able to induce degranulation, the introduction of HER2 IgE could cause systemic anaphylaxis when MCs, basophils and other effector cells decorated with the antibody encountered the soluble antigen. To further ensure that systemic anaphylaxis would not be likely in a human application, an *in vivo* mouse model was employed. Using this model, it was shown that ECD^{HER2} was not able to induce local anaphylaxis. In the mouse model used, the endogenous gene for the alpha subunit of FcεRI was removed and replaced with the human sequence of FcεRIα to allow binding of the human IgE to mouse effector cells. It should be noted that while MCs and basophils in this model are able to bind the human IgE via the FcεRI receptor, other effector cells that interact with IgE via the low affinity CD23 receptor are not able to bind IgE, thus limiting the predictive ability of this model. Despite its possible limitations, the authors were able to show that administration of C6MH3-B1 was able to prolong the lifespan of mice bearing HER2 positive human breast cancer cells as compared to control IgE. Finally, a very small study was performed in the non-human primate model system *Macaca fascicularis*, with two monkeys being injected with either low or high concentrations of C6MH3-B1 and subsequently monitored for overall health

and eating habits and the serum concentrations of human IgE were measured after one week. The animals in the study displayed no adverse effects of treatment and human IgE levels returned to normal by the one-week observation point. While this study was very limited and would need to be expanded upon before conclusive statements can be made about the safety of C6MH3-B1, it is nevertheless encouraging.

CHAPTER III

DIFFERENTIATION OF MAST CELLS FROM ADIPOSE DERIVED STEM CELLS

III.1 Introduction

Adipose Derived Stem Cells (ADSCs) were first identified in 2001 as mesenchymal-type adult stem cells present in the stromal vascular fraction (SVF) of human liposuction aspirate [82]. Their presence was hypothesized due to the previous observation of mesenchymal stem cells in connective tissues of other vertebrate groups as well the fact that adipose tissue is derived from the same embryonic tissue (mesoderm) as bone marrow, the previous source of autologous adult stem cells. The aim of the study in which ADSCs were initially described was to develop an alternative source of autologous stem cells for tissue engineering given the highly invasive and painful nature of bone marrow extraction. The common wisdom at the time of their discovery was that the differentiative capacity of ADSCs were limited to the classical four normally associated with mesenchymal stem cells, namely adipogenic, osteogenic, chondrogenic and myogenic. In the years since the identification of ADSCs, researchers have successfully differentiated them into cell types of both endodermal and ectodermal origin. MCs are a difficult cell type to study in the laboratory setting; the reasons for this are twofold. The first reason is that they are difficult to obtain in large numbers from human donors. MCs are most commonly isolated from skin removed during surgical procedures. This process

is costly and inefficient, and the resultant cells take months to grow to a number sufficient to perform experiments. The second contributing factor to the difficulty of the study of MC biology in humans is the significant difference that exists between human and murine MC biology [149], [150]. The fundamental requirement for a model system is that it faithfully recapitulates the functions and phenomena one wishes to investigate, a requirement that is not satisfied by either mice or rats in regard to MCs and mechanisms underlying allergic disease. The difficulty posed by these two factors has limited the understanding of the role of MCs in both normal and diseased states of human physiology.

The purpose of this investigation was to test the hypothesis that ADSCs obtained from human adipose tissue could be used to differentiate into MCs as a new source to study MC biology. To this end we employed the SVF isolation method previously described and developed a protocol using morphogens produced by classically-derived human skin mast cells (hSMCs). The resultant cells were investigated using various metrics to confirm their identity as MCs.

III.2 Methods

III.2.i ADSC Isolation

Adipose tissue was obtained in two forms, as bulk resected adipose (BA) tissue and as liposuction aspirate (LA). Liposuction aspirate was obtained locally from a plastic surgeon, whereas whole tissue was obtained from the Cooperative Human Tissue Network (UNCG IRB 12-0199). Adipose tissue was first washed in 1x Phosphate buffered saline with 1% penicillin/streptomycin and 1% amphotericin B to remove red blood cells. In the case of resections, tissue was homogenized mechanically using surgical shears to mince tissue and break up connective tissue. LA is mechanically homogenized during the liposuction procedure and therefore does not require as extensive mechanical homogenization or mincing as does BA. Thirty milliliter aliquots of homogenized tissue were placed in 50 mL tubes and digested in roughly 20 mL of 0.1% Collagenase Type IA in wash buffer (1x Hanks Buffered Salt Solution, 0.04% $NaHCO_3$, 1% FBS, 10 mM HEPES, 0.5% Amphotericin B, 1% penicillin/streptomycin) for 90 minutes at 37°C under constant agitation. Following digestion, the dissociated cell slurry was filtered through sieves to remove undigested tissue then centrifuged at 360 x g for 15 minutes to fractionate the slurry into a lipid-laden adipocyte layer, a turbid aqueous layer, and the cell pellet containing the SVF (**Figure 3.1**). The adipocyte rich phase and all but 10 mL of the aqueous layer were removed and discarded. The remaining cell pellets were resuspended in fresh wash buffer and aliquots were pooled and centrifuged again at 360 x g for 10 minutes. The second centrifugation yields only a cell pellet and aqueous phase

which is removed and discarded. The remaining cell pellet is then plated in a tissue cell culture flask of varying size depending on the volume of tissue processed.

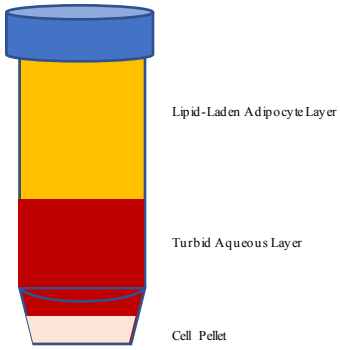

Figure 3.1. Diagram of Fractionated Adipose Tissue. This figure shows the approximate proportions of each of the three phases produced by centrifugation of adipose cell slurry. The cell pellet contains the stromal-vascular fraction of adipose tissue.

Cells were plated and grown for a minimum of one week in ADSC media (Dulbecco's Modified Eagles Medium, 4.5g/L glucose, 10 mM HEPES, 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin/amphotericin B) at 37°C under a 5% CO_2 atmosphere. Media was changed once or twice per week and flasks were maintained in ADSC media until they reached confluence. Once cells reached confluence they were used for various applications including cryopreservation, characterization or differentiation into MCs as discussed below. ADSC cultures can be sub-cultured and expanded into a larger number of flasks, however this was not generally done as ADSC differentiative capacity is affected by passage number [118].

III.2.ii ADSC Characterization

ADSCs were characterized using both transmitted and fluorescent light microscopy. Transmitted light microscopy was performed using an inverted microscope with phase contrast and collected using a CCD camera. For fluorescent microscopy, a Zeiss spinning disc confocal microscope was used to collect images. ADSCs were stained with MitoTracker™ Red CMXROS or MitoTracker™ Green FM for 30 mins, per the manufacturer's instructions for time lapse experiments and were imaged unstained to analyze the autofluorescent properties of ADSCs.

Immunophenotyping of cell surface receptors was performed using flow cytometry. Cells were first removed from flasks via incubation with Corning™ CellStripper Dissociation Reagent at 37°C under a 5% CO_2 atmosphere until cells released from the plastic. Cells were pelleted by centrifugation, washed with 1x Tris buffered saline (TBS) and resuspended in 1x TBS with 1:400 ratio of normal goat serum for 2 hours in order to block non-specific binding. Directly conjugated primary antibodies were purchased from BD Biosciences and used per manufacturers recommendations. The antibodies used and the conjugation of each is shown in **Table 3.1**.

Table 3.1. Antibodies Used for Flow Cytometry. The five antibodies listed above were used in the flow cytometric immunophenotyping of ADSCs.

Marker	*Conjugation*
CD 105	FITC
HLA-DR	FITC
HLA-ABC	FITC
CD 14	PE
CD 44	PE

Flow cytometry was performed using a FACSAria™III flow cytometer equipped with a 70 μm nozzle. Detector voltages were set using unstained ADSCs as a negative control and the threshold was set automatically by the instrument software. At least 100,000 events were recorded for analysis.

III.2.iii Mast Cell Differentiation

hSMC are grown in X-Vivo™ 15 media supplemented with 80 ng/mL of recombinant human stem cell factor (rhSCF). After a minimum of one week in culture, the media is removed from the hSMCs; this media is referred to as conditioned media. The conditioned media is then spiked with a 20 ng/mL human IgE, 20 ng/mL rhSCF and passed through a 0.22 μm filter to remove any cells that may have been inadvertently collected during the removal of media from the hSMC cultures. This filtrate is the final complete MC differentiation media. After culture of ADSCs in ADSC media for a minimum of one week, ADSCs were cultured in complete MC differentiation media for the remainder of the investigations. ADMCs could be collected a minimum of two weeks

after addition of differentiation media. Collection was often delayed a further two weeks to allow cultures to produce sufficient numbers of ADMCs for subsequent investigations.

III.2.iv Morphological and Histological Characterization of ADMCs

ADMCs were characterized morphologically with transmitted light microscopy using an inverted microscope as used to image ADSCs. ADMCs were further characterized histologically using toluidine blue staining. ADMCs were collected by removing media from flasks and centrifuging them to pellet the cells. Pelleted cells were resuspended in 200 μL of 1x PBS, applied to CytoSpin™ funnels and spun for 5 minutes at 600 rpm in a specialized centrifuge. Slides were then air dried and fixed in methanol for one hour. Fixed slides were washed and stained for at least two hours at room temperature with a 0.5% toluidine blue solution made with 0.5 N HCl. Following staining, slides were washed to remove unincorporated dye. Slides were mounted using Permount™ Mounting Medium and cover slipped with 1.5 thickness cover slips and mounting medium was allowed to cure for 15 mins. Cured and mounted slides were viewed on an inverted light microscope and images were collected using a CCD camera. ADMC were identified by their characteristic heterochromatic staining of the granules. Size measurements were determined using software to convert pixel number to length in micrometers.

III.2.v Immunohistochemical Characterization

ADMCs were cytocentrifuged onto glass slides and fixed using a CytoSpin™apparatus and methanol as described above. Following methanol fixation for one hour, cells were washed twice with 1x PBS then blocked with 1x PBS/5% bovine serum albumin (BSA) for 30 minutes. Primary antibody staining was performed overnight in dark, humid chambers at 4 °C. Slides were washed twice, then stained with fluorescently labeled secondary antibody for two hours, with the Hoechst 33342 being added for the final 20 minutes. Labeled slides were washed with 1x PBS once and mounted in mounting media and imaged using a Zeiss AXIO Observer Z1 Spinning Disc Confocal Microscope.

III.2.vi Phenotypic Characterization of ADMC

RNA was extracted from ADMC using the Qiagen RNeasy Plus Mini kit per the manufacturers recommendation. Reverse transcriptase PCR (RT-PCR) was performed using the Qiagen OneStep RT-PCR kit using primers for β-actin, tryptase, chymase, c-Kit, and FcεRIα listed in **Table 3.2**. Cycling conditions were: 50°C for 30 minutes, 95°C for 15 minutes, followed by 35 cycles of 94°C for 45 seconds, 53-63°C for 45 seconds (according to primer T_m), 72°C for one minute and a final 10-minute extension at 72°C. Upon completion of PCR, samples were applied to a 1% agarose gel containing ethidium bromide (EtBr) to allow visualization of DNA. The gel was run for 1 hour at 65 Volts and imaged using an agarose gel transilluminator with appropriate filters for ethidium bromide.

Table 3.2. Primer Sequences Used for RT-PCR of ADMCs. RT-PCR was performed on RNA collected from ADMCs to determine the levels of expression of four MC specific transcripts.

Gene	Direction	Sequence (5'→3')	Tm (°C)
FcεRI-α	Forward	CCT TGA ACC CTC CAT GGA ATA G	55.1
	Reverse	CAC TTT GCC CGT ACA GTA GTA G	55
c-Kit	Forward	TCT ATG CTC TCGCAC CTT TC	54.5
	Reverse	GAC TCA TGG GCT TGG GAA TA	54.4
Tryptase	Forward	GCA AAA TAC CAC CTT GGC GCC TAC ACG G	65.1
	Reverse	GTG ACA CGG GTG TAG ATG CCA GGC	63.8
Chymase	Forward	CCT GCT GCT CTT TCT CTT GT	55
	Reverse	GCT CCA AGG GTG ACT GTT ATA G	54.9
β-Actin	Forward	AAT GTG GCC GAG GAC TTT GAT TGC	60.2
	Reverse	AGG ATG GCA AGG GAC TTC CTG TAA	59.9
18s Ribosomal Subunit	Forward	GCC GCT AGA GGT GAA ATT CT	54.8
	Reverse	TCG GAA CTA CGA CGG TAT CT	54.8

III.2.vii Cell Surface Expression of Mast Cell Markers

Surface receptor characterization of ADMCs was performed using flow cytometry. Cells were recovered by centrifugation at 4°C, washed with 1x PBS/1% BSA, and blocked for 30 minutes at 4°C with a 1:400 dilution of normal goat serum. The cells were washed and incubated with 5 μg/ml of mouse anti-human FcεRI, c-Kit, or the mouse control IgG MOPC (Sigma) for two hours at 4°C. After antibody (Ab) labeling, the cells were washed and incubated with a 1:100 dilution of $F(ab')_2$ -FITC-goat anti-mouse Ab (Jackson Immunology) for 30 minutes at 4°C. After three washes, cells were

re-suspended in 400 μL of 1x PBS. The mean intensity of fluorescence was determined for at least 10^5 cells using a FACSAria™III (BD Biosciences). Detector voltages were set using unstained ADMCs and the threshold was set automatically by the instrument software. At least 100,000 events were recorded for analysis.

III.2. viii Functional Characterization

In order to determine the functionality of ADMCs, preformed and newly synthesized mediator release was measured using the β-hexosaminidase release assay and cytokine specific enzyme linked immunosorbent assay (ELISA). Release of preformed β-hexosaminidase (a surrogate for histamine with similar release kinetics) was determined by measuring the amount of a chromogenic substance produced after incubation with an engineered β-hexosaminidase substrate. Briefly, ADMCs were activated in a 37°C water bath for thirty minutes using 1 μg/mL of the FcεRI crosslinking antibody 3B4 in X-Vivo™ 15. Following activation, cells were pelleted, supernatant was removed, and aliquots were loaded onto a multiwell plate. The remaining cell pellet was then resuspended and incubated overnight to facilitate measurement of secondary cytokine production. The chromogenic, β-hexosaminidase substrate para-Nitrophenyl N-acetyl-β-D-glucosaminide (PNP) was added to the cell supernatant and incubated for 90 minutes. Reactions were terminated by the addition of glycine. The amount of converted substrate was quantified spectrophotometrically by measuring the absorbance of the reaction at 405 nm. In order to determine what percentage of the total intercellular store of β-

hexosaminidase is released, total β-hexosaminidase levels are measured in cell lysates produced by three successive freeze/thaw cycles at -80°C and 37°C, respectively.

Enzyme linked immunosorbent assay was used to measure newly synthesized cytokines generated in response to FcεRI challenge. Following overnight incubation, cells were pelleted, and supernatants were collected and ELISA was performed as described previously [186]. Capture antibodies were immobilized onto plastic multiwell plates per manufacturers recommendations in binding buffer. Aliquots of ADMC supernatants were loaded into wells with immobilized capture antibody and binding was allowed to proceed. Following incubation, wells were washed, and detection antibody was added, incubated then washed. Secondary antibodies conjugated with horseradish peroxidase (HRP) specific for the detection antibody was used in conjunction with a chromogenic substrate. Measurements were taken using a spectrophotometer and concentrations in samples were calculated using a standard curve run with each experiment.

III.3 Results

III.3.i ADSC Isolation and Characterization

Human adipose tissue was obtained from two sources and processed as described above. **Table 3.3** shows the recorded amounts and sources of adipose derived stem cell isolation performed. All tissue used in the present study was taken from female subjects, the majority of which identified as African American. The average weight of bulk resected adipose tissue processed for this study was 195 grams and the amount of

liposuction aspirate ranged from 200 mL at the lowest to 1 L at the highest. The number of flasks seeded after each preparation was generally 4 T-50 flasks, however smaller preps were seeded into fewer flasks.

Table 3.3. Characteristics of Adipose Tissue Processed. Data provided with each shipment of adipose tissue was recorded. Data not available is shown as a question mark.

Bulk Resected Tissue	date	Mass	Age	Sex	Race	Flasks Seeded
	8/4/16	50.6	59	F	B	4
	8/25/16	160	32	F	B	4
	9/28/17	?	?	?	?	3
	10/6/17	341	?	?	?	?
	11/1/17	147	?	?	?	?
	1/3/18	239	39	F	W	4
	2/23/18	200	46	F	B	2
	3/14/18	288	59	F	W	?
	3/22/18	100	38	F	B	2
	4/12/18	226	43	F	W	3
Liposuction Aspirate	**Date**	**Volume**	**Age**	**Sex**	**Race**	**Flasks Seeded**
	2/27/17	700 mL	?	?	?	4
	9/27/17	?	?	?	?	4
	4/5/18	1L	58	F	?	4
	4/9/18	200 mL	?	?	?	2

All adipose tissue used was normal, but the overall health of the donor was not known. It can be presumed, however, that many of the subjects were obese in the case of liposuction aspirate donors.

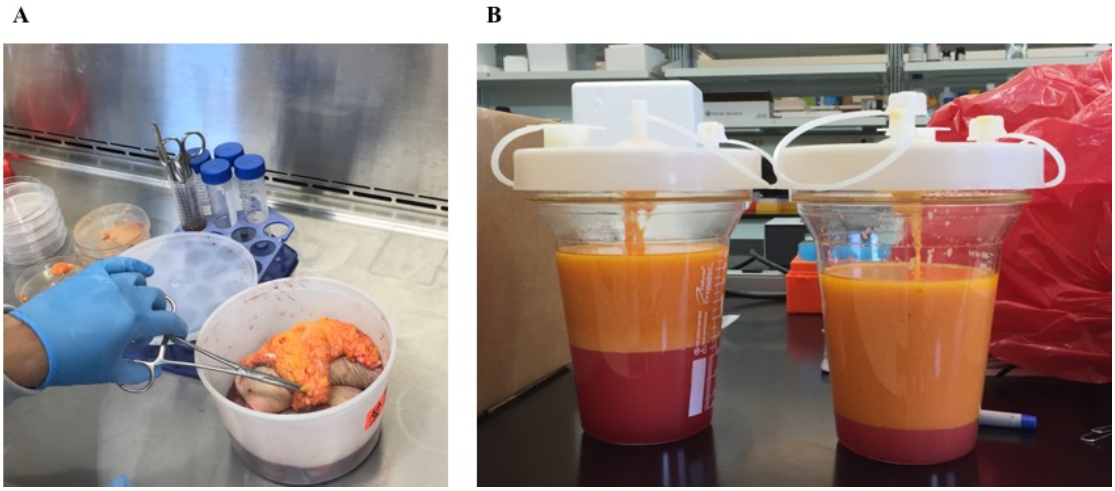

Figure 3.2. Formats of Adipose Tissue Used for ADSC Isolation. Bulk adipose tissue (A) was obtained from the Cooperative Human Tissue Network and transported in sterile tubs to site of cell isolation. Liposuction aspirate (B) was obtained from local plastic surgeons and transported in collecting vessels used during surgery.

Figure 3.2 shows the appearance of the two types of tissue upon receipt in the laboratory. After mechanical homogenization, enzymatic digestion and washing, freshly plated cells grew as adherent fibroblast-like cells as seen in **Figure 3.3**.

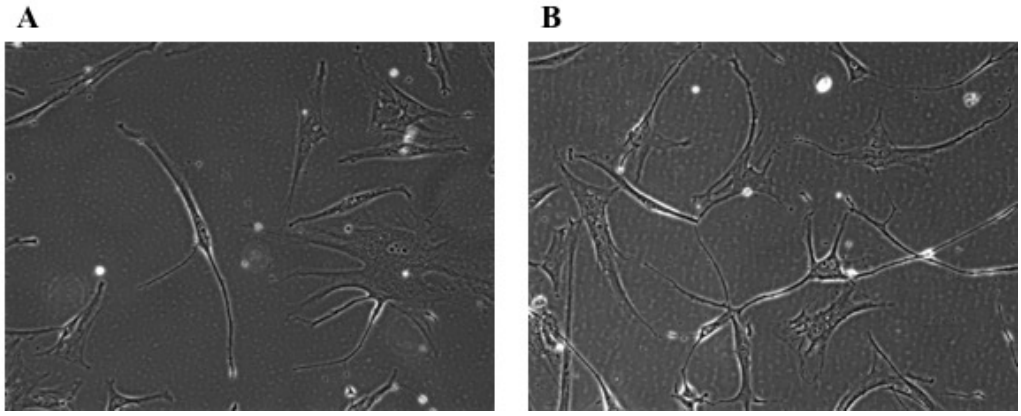

Figure 3.3. ADSC Morphology. Light micrographs (A-B) showing ADSC morphology. Images obtained using 10x objective and inverted light microscope under phase contrast.

Cells reached confluence on the flask bottom within approximately three days of initial seeding. ADSCs exhibited varying degrees of contact inhibition as evidenced by the presence of both epithelial-like cell monolayers and clumps of cells growing on top of one another **(Figure 3.4)**.

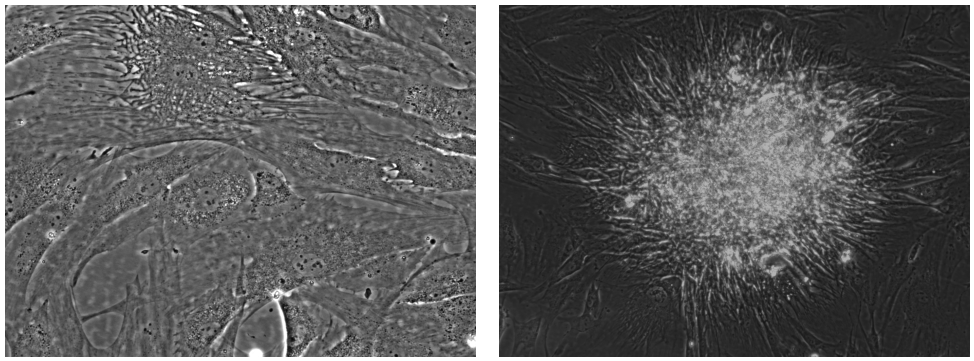

Figure 3.4. ADSCs are Heterogeneous with Respect to Contact Inhibition. ADSCs were isolated as previously described from bulk adipose tissue and cultured for 7 days. ADSCs grew in monolayers (Left) and also formed clumps (Right). Magnifications are 40x (Left) and 10x (Right).

Cell morphologies of ADSCs are highly complex and varied. As seen in **Figure 3.5**, several cell types differing in morphology, or ADSCs at different stages of differentiation, were observed in ADSC cultures. These included a myocyte-like phenotype (**A**), large, rounded yet still epithelial cells (**B**), highly spread out cells with what appear to be mitochondrial networks observable in unstained cells (**C**) and a cuboid, endothelial-like phenotype cell that formed "nests" that were distinct from surrounding fibroblast-like cells (**D**).

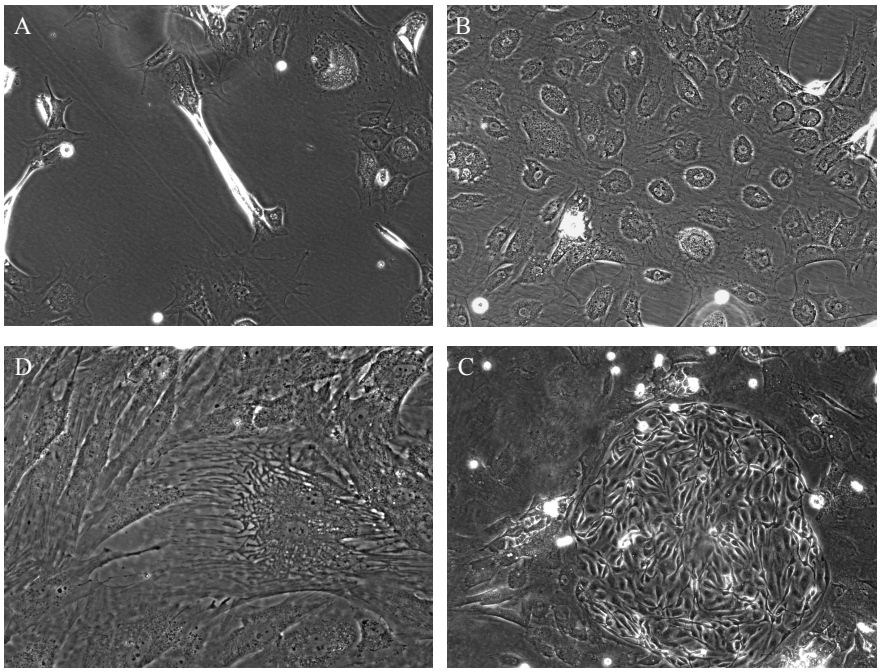

Figure 3.5. Phase Contrast Microscopy of ADSC Cultures. In addition to the canonical fibroblast-like morphology of ADSCs other morphologies are observed including myocyte-like (A), rounded (B), internally complex (C) and endothelial-like (D). Magnifications were 10x (A-B, C) and 40x (D).

Due to the impediments to morphological identification of ADSCs, other markers of stem cells were examined such as the tendency of stem cells to exhibit strong autofluorescence. As seen in **Figure 3.6**, ADSCs exhibit a range of autofluorescence from very strong, as seen in the cell clump (**A)** to moderate (white arrows) or none at all (red arrows) (**B**) when illuminated with 488 nm light and monitored for the emission of 512 nm light. This autofluorescence is most likely the result of one of the following types of molecules: NADH [187], lipofuscin [188], or riboflavin [189]. The autofluorescence of ADSCs seems to decrease upon induction of differentiation and loss of "stemness"

however this assertion is based on personal observation and must be tested empirically before autofluorescence can used as a marker of ADSC differentiation.

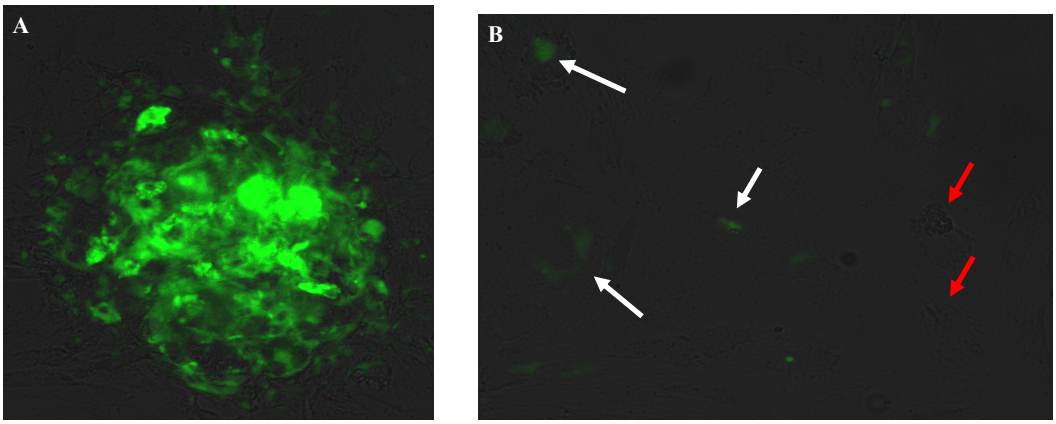

Figure 3.6. Autofluorescent Properties of ADSCs. ADSC were cultured for 7 days. Images were collected using a standard fluorescent microscope using standard FITC excitation and emission settings (488/512 nm). Large clumps of cells (A) showed the highest levels of autofluorescence. ADSCs were heterogeneous with respect to autofluorescence (B), white arrows denote autofluorescent ADSCs and red arrows denote ADSCs that do not show autofluorescence. Magnifications were 20x (A) and 4x (B).

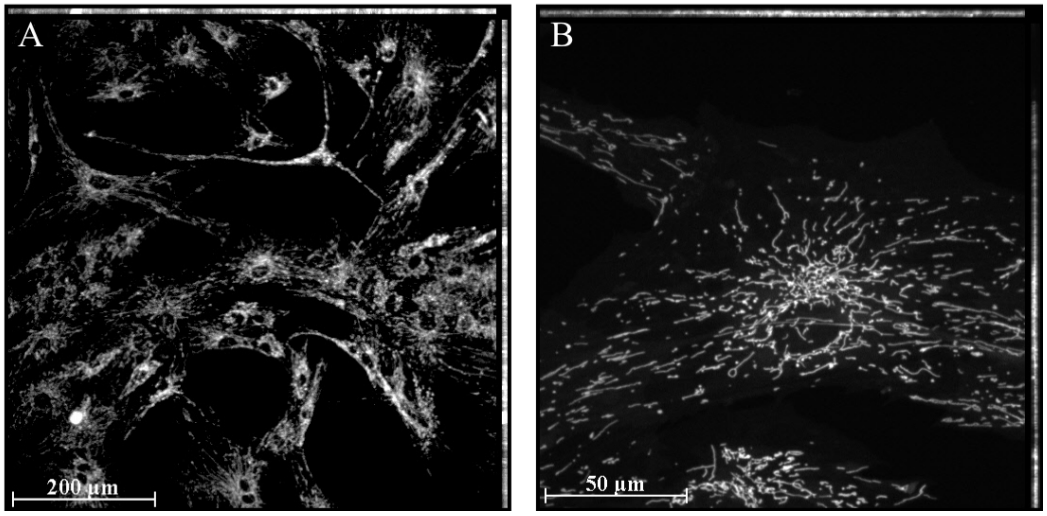

Figure 3.7. Mitochondrial Staining of ADSCs. ADSCs were stained with MitoTracker™ Red. Images were collected at 10x (A) and 40x (B) magnification. Mitochondria showed a tubular morphology.

ADSCs were stained with MitoTracker™ Red CMXROS and time lapse images were taken for 24 hours. **Figure 3.7** shows a representative image of one ADSC. ADSCs were motile and displayed mitochondrial streaming consistent with a metabolically active cell. Mitochondria were observed to be evenly distributed throughout the cell displaying a tubular morphology.

Further immunophenotypic characterization of the ADSC was performed using flow cytometry. **Figure 3.8** shows density plots obtained from an ADSC experiment. Quadrants were determined to identify cells positive (High Fluorescence, lower right quadrant) and cells negative (Low Fluorescence, lower left quadrant) for antibody staining. **Figure 3.9** shows histograms created by gating on the positive population of each condition. The ADSC phenotype was determined to be: $CD44^{+}CD14^{-}CD105^{-}$ and $HLA\text{-}ABC^{+}$.

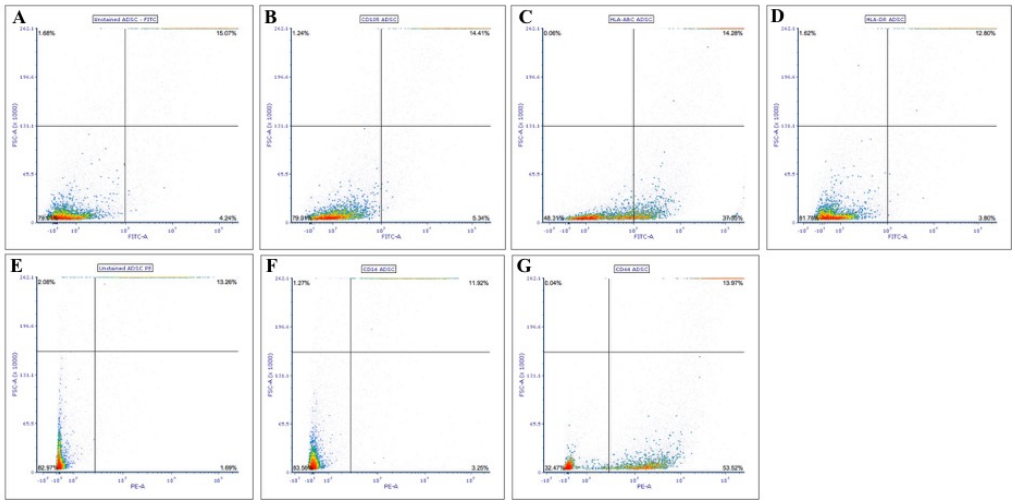

Figure 3.8. ADSC Immunophenotypic Characterization. Quadrants were determined using unstained cells (A) where the lower left quadrant contains the negative population and the bottom right the positive population.

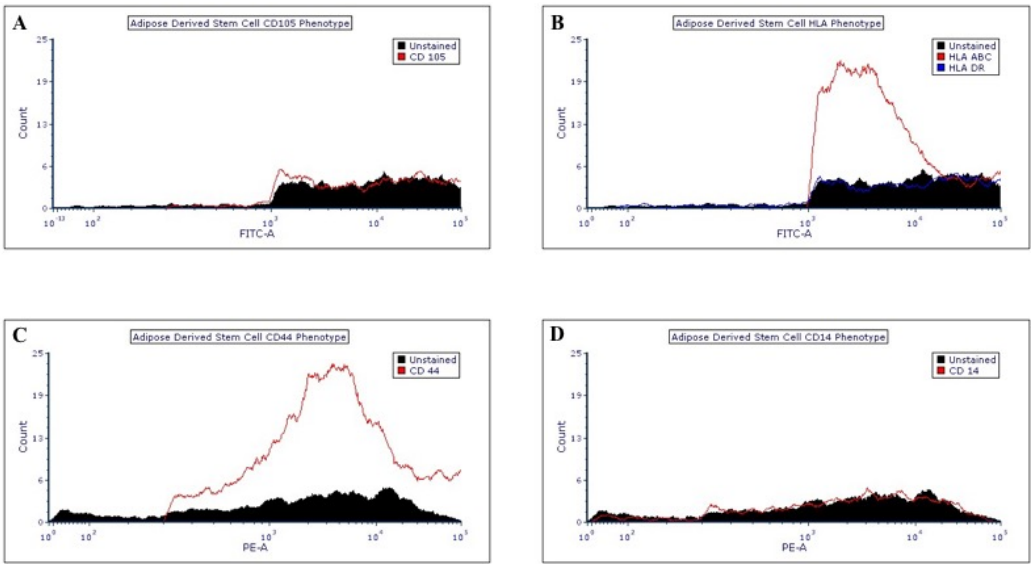

Figure 3.9. ADSC Immunophenotypic Characterization - Gating. Histograms shown are gated to remove the negative population as determined by the unstained control above.

III.3.ii ADMC Differentiation and Characterization

ADSCs were transitioned from ADSC media to complete MC differentiation media after a minimum of one week of culture and media was changed approximately once per week. Cultures generally started to produce MCs after approximately two weeks in differentiation media, however MC production generally started at a relatively low rate, which subsequently increased in roughly another two weeks of culture. During the initial stages of differentiation, MC-like cells begin to form as large rounded cells with visible cellular protrusions that appear to form anchors to the surrounding cells as shown in **Figure 3.10**. It is not clear if the initial MCs arise by budding off adherent cells attached to the plastic or if the attached cells themselves release from the tissue culture plastic to become MCs.

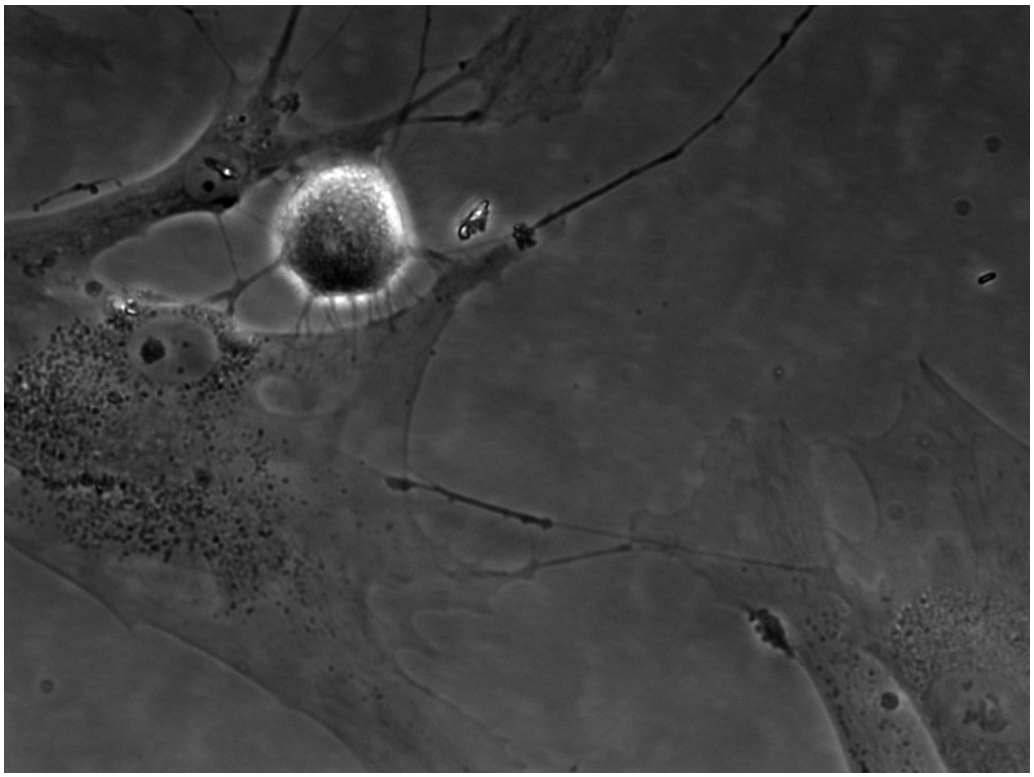

Figure 3.10. Phase Contrast Microscopy of ADSCs Incubated in MC Differentiation Media for 14 days. After 14 days small numbers of granulated, spherical cells began to appear. As shown, cells were not fully detached from the substrate, but were attached via extensions of the cell membrane to surrounding cells. Magnification used was 40x.

ADMCs were observed to slough off of large aggregates of cells (**Figure 3.11)** that formed in ADSC cultures after addition of differentiation media.

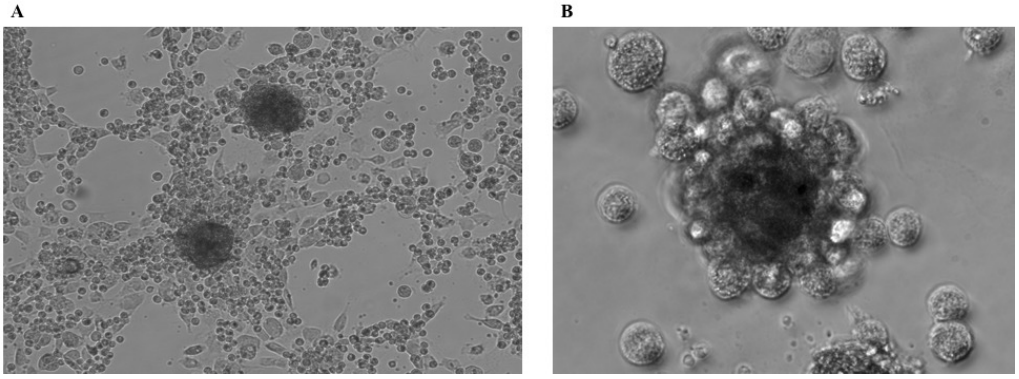

Figure 3.11. ADMC. ADMCs in culture growing on monolayer of ADSCs (A) are seen growing in clumps. One clump shown in higher magnification (B). Images obtained using a 5x objective (A) and a 40x objective (B) and inverted light microscope.

The ADMC showed a morphology similar to that previously described for human skin MCs (connective tissue, MC_{TC} [190]). Specifically, ADMC showed a highly spherical morphology with dense, complex granules. As seen in **Figure 3.12**, the sizes of MCs as measured by determining the approximate diameter, showed a large degree of variation with the largest cell measuring 27.2 μm in diameter and the smallest cell measuring 16 μm in diameter. The average diameter was 21.28 μm ± 3.61. The cause of this variation has not yet been determined.

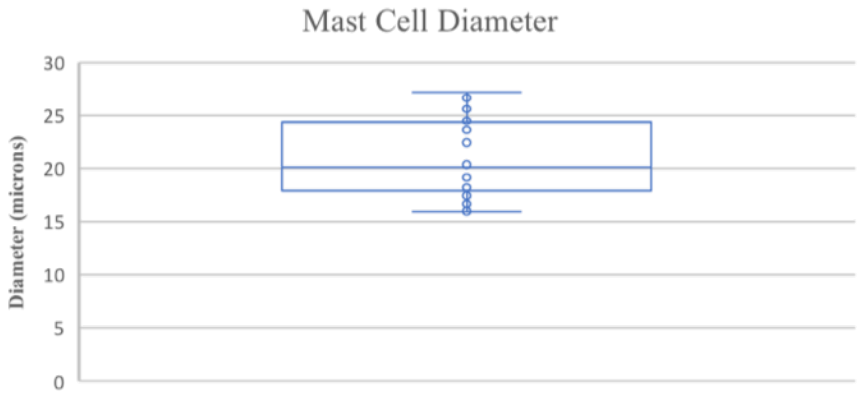

Figure 3.12. Size Distribution of ADMCs. The diameters of 44 ADMCs were measured and are displayed as a box and whisker plot.

As seen in **Figure 3.13**, toluidine blue staining yielded metachromatic granule staining characteristic of MCs. Some variation was observed in the degree of toluidine blue staining, which is likely due to the somewhat heterogeneous nature of the MCs produced using this protocol.

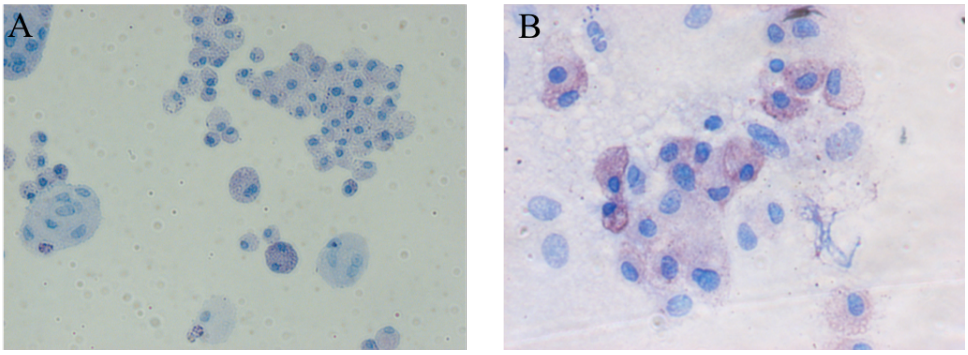

Figure 3.13. Toluidine Blue Staining of MCs. ADMC (A) showed characteristic staining similar to hSMC (B) with some variation in the intensity of toluidine blue staining. Magnifications are 10x (A) and 40x (B).

A two step, reverse-transcriptase polymerase chain reaction (RT-PCR) protocol was used to measure the expression of four MC specific genes: tryptase, chymase, FcεRI-α, and c-kit. In order to restrict the pool of amplified RNA to mature mRNA, a first-step cDNA synthesis was performed using Oligo-dT primers. Oligo-dT primers used in first strand cDNA synthesis recognize the poly-adenylated tails of fully processed mRNA, removing the otherwise complicating unprocessed or non-coding RNAs. cDNA produced in this way was then used for traditional PCR using primers designed to amplify small (<1000 base pairs) specific portions of mRNA corresponding to each gene. Housekeeping genes used as loading controls were β-actin and the 18s ribosomal subunit. As seen in **Figure 3.14**, expression was observed for all six genes tested. Expression of tryptase and chymase mRNA was lower than that of the other four genes tested, however not below the limit of detection. Furthermore, there is considerable debate in the literature over the "normal" levels of several MC specific transcripts including and especially chymase. This is even further confounded by the fact that there are many sub-types of MCs, all of which express different compliments of genes and at different levels.

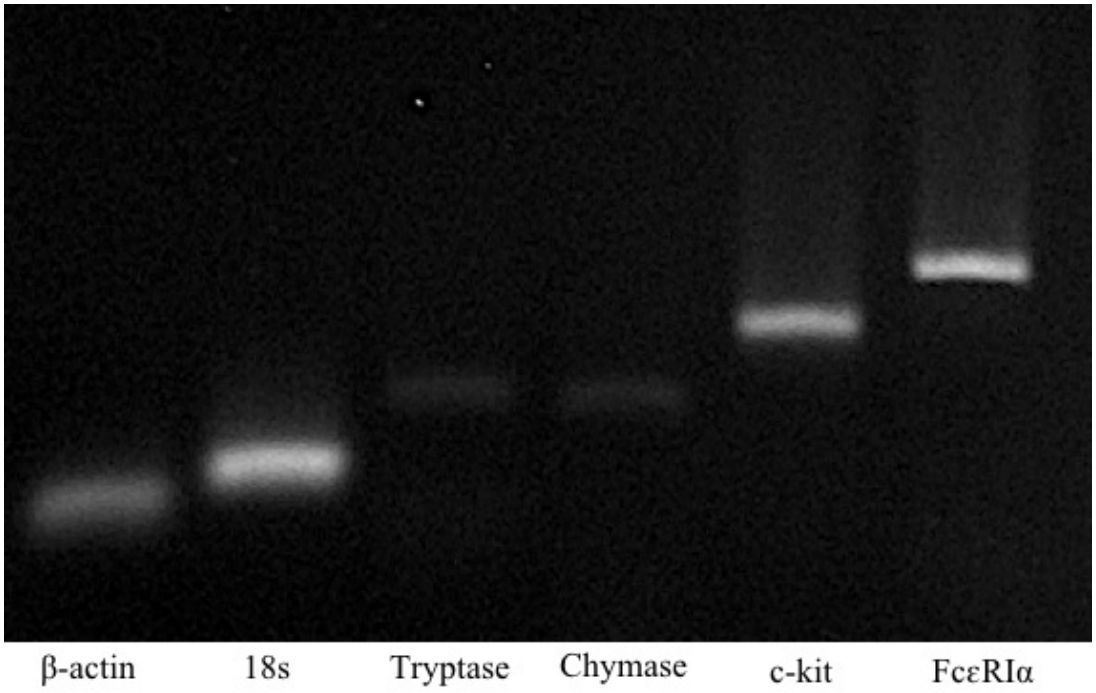

Figure 3.14. ADMC Gene Expression. Total RNA was extracted from ADMCs and expression of four MC specific genes were analyzed. Two housekeeping genes were also measured as controls.

Immunohistochemistry verified the expression of tryptase and chymase (**Figure 3.15)** suggesting the ADMC are similar to connective tissue MCs or MC_{TC} [191]**.** Both proteins showed characteristic intense punctate staining, indicative of highly concentrated secretory vesicles or granules most commonly associated with MCs. In agreement with the observation of varying levels of staining produced by toluidine blue, both tryptase and chymase showed considerable cell-to-cell variation in expression levels.

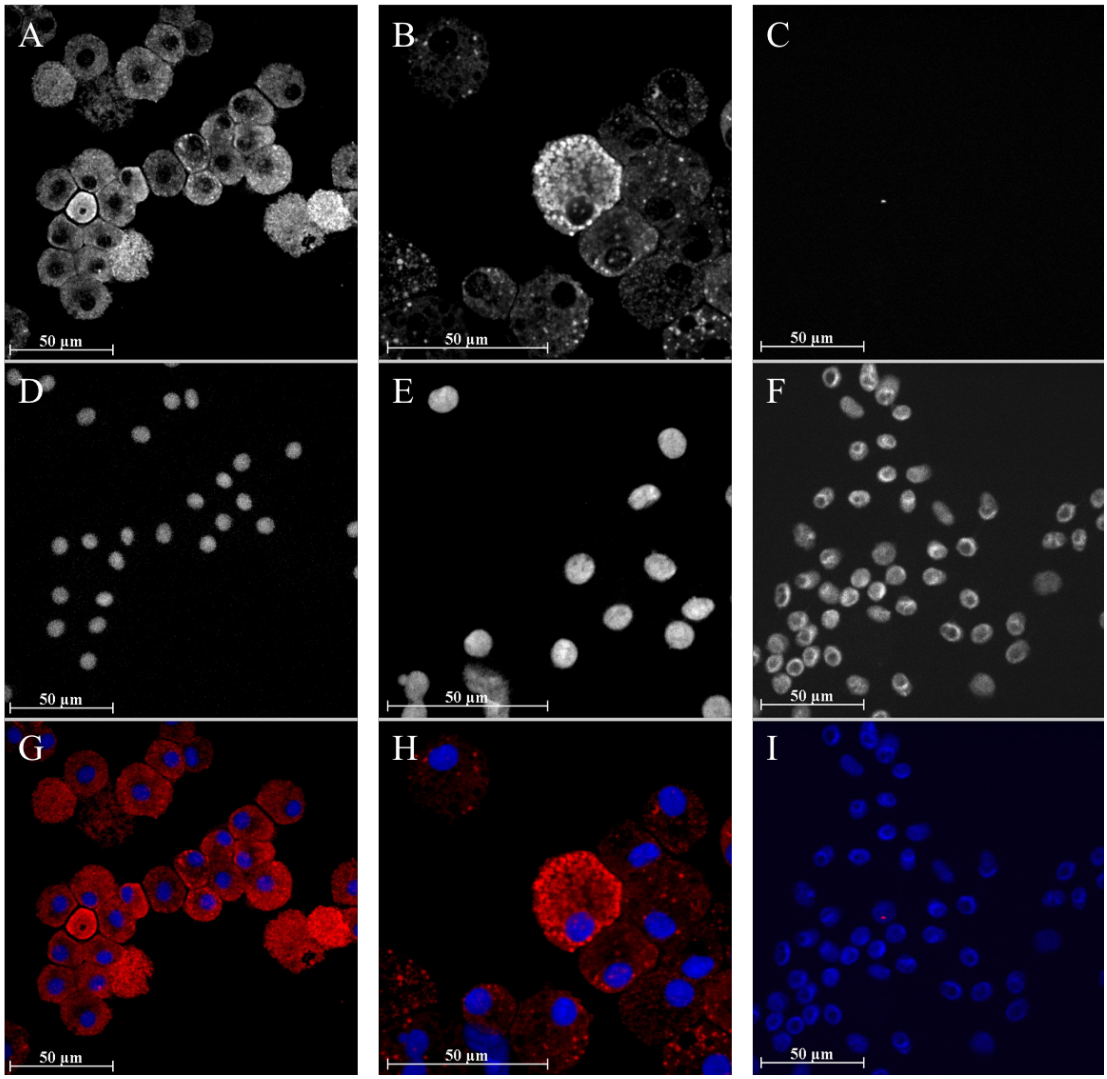

Figure 3.15. Immunohistochemical characterization of ADMC. ADMC were stained using antibodies specific to human tryptase (A) or chymase (B). Secondary antibody was labeled with Cy3 and nuclei were stained with Hoechst 333342.

Flow cytometric analysis was performed to measure both the presence of cell surface markers of MCs and the proportion of cells in ADMC cultures that express them. As seen in **Figure 3.16**, ADMCs showed positive surface expression of FcεRI, c-kit and HLA-ABC and no surface expression of HLA-DR. In the case of FcεRI, several

antibodies were tested which gave similar, although not identical expression levels. This is not surprising given that these antibodies recognize different epitopes on the FcεRI molecule which are likely to exhibit varying degrees of accessibility to the antibody under the experimental conditions used. HLA profiles of MCs previously reported were in agreement with the profile determined for ADMC, namely, ABC^{+} [192] and DR^{-} [193].

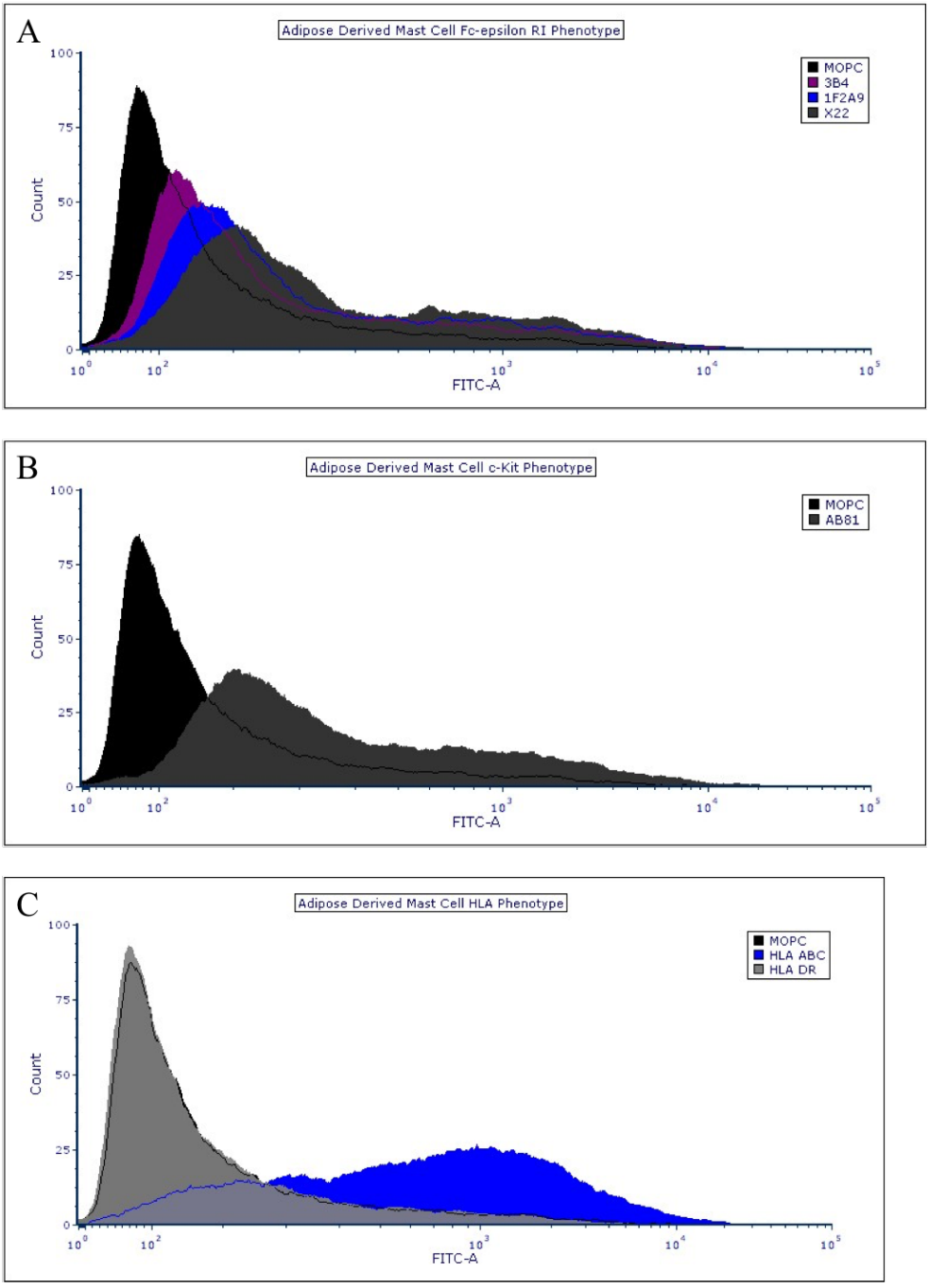

Figure 3.16. ADMC Immunophenotypes. ADMCs were stained with antibodies specific for the canonical MC surface receptors c-kit (C) and FcεRIα (A). HLA phenotype (B) was determined using antibodies for two classes of HLA molecules.

Functional characterization was performed in response to FcεRI-dependent and FcεRI-independent stimuli. As shown in **Figure 3.17**, ADMC released roughly 40% of total β-hexosaminidase in response to FcεRI challenge, in comparison to almost 80% for the skin derived MCs. ADMCs also had considerably higher levels of spontaneous release of β-hexosaminidase as compared to their skin derived counterparts. ADMCs were also analyzed for their ability to degranulate in response to non-FcεRI challenge. As seen in **Figure 3.18**, ADMC degranulation was induced by poly-L lysine and the calcium ionophore A23187, however not by lipopolysaccharide (LPS) or the peptide fMLP.

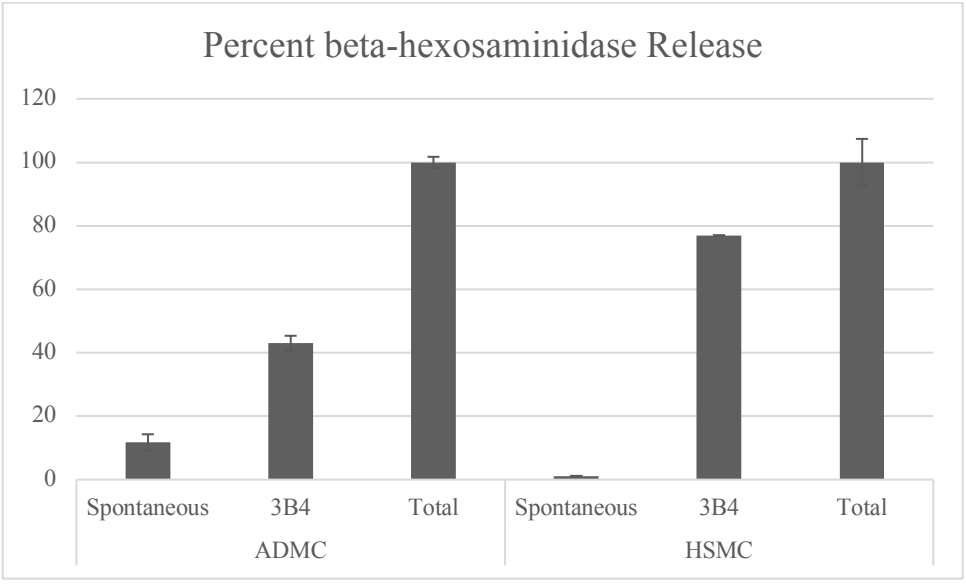

Figure 3.17. β-hexosaminidase Release from Stimulated MCs. ADMC and hSMCs were compared for their ability to degranulate in response to FcεRI aggregation by the antibody 3B4.

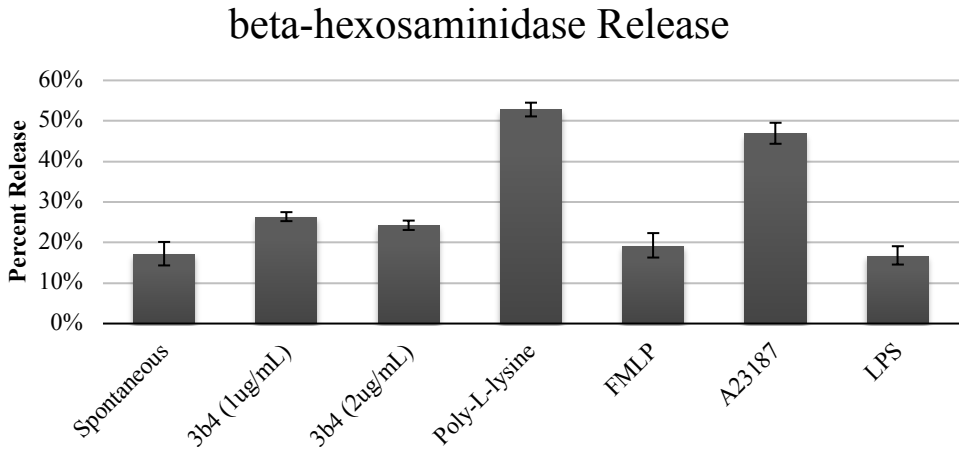

Figure 3.18. Non-IgE Mediated Degranulation. β-hexosaminidase release was measured from ADMC in response to various non-IgE activators.

In order to determine whether or not secondary cytokine production could be induced, granulocyte/macrophage-colony stimulating factor production was measured using ELISA. As seen in **Figure 3.19**, ADMC were stimulated to release the contents of their granules upon crosslinking of FcεRI by the antibody 3B4. Spontaneous release of GM-CSF was not detectable for skin derived MCs as compared to roughly 1400 pg/mL in ADMCs. 3B4 induced GM-CSF production in hSMCs was roughly 4800 pg/mL as compared to 3000 pg/mL for adipose derived MCs. Considerable variation was observed in the measurements taken for ADMCs and the difference between spontaneous and 3B4 induced GM-CSF production that did not reach statistical significance ($P = 0.12$). The spontaneous production of GM-CSF by ADMCs was found to be statistically significantly ($P=0.03$) higher than that of hSMCs.

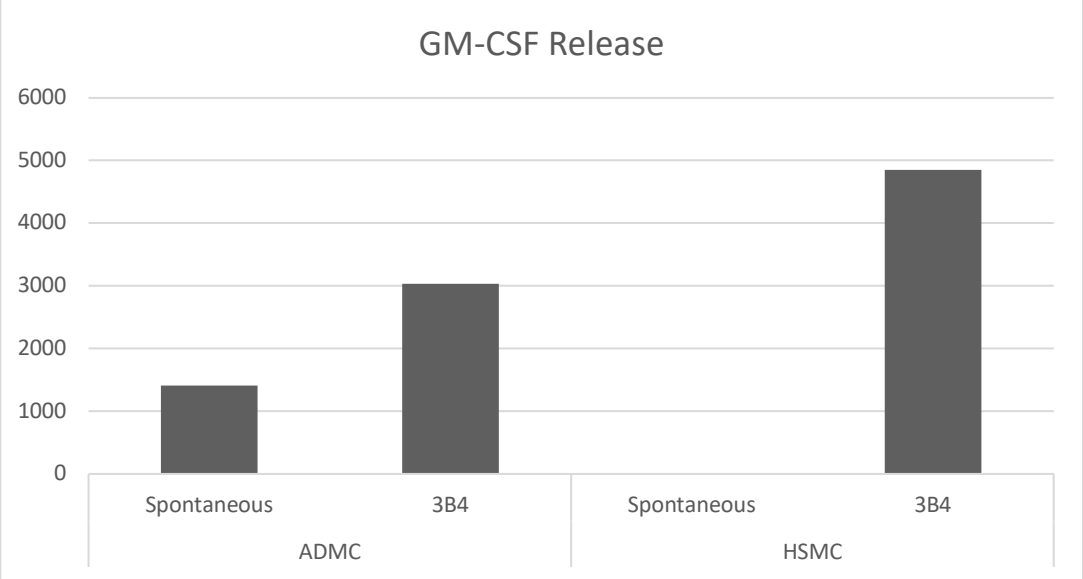

Figure 3.19. GM-CSF Production in Stimulated MCs. GM-CSF production was measured by ELISA in ADMCs and hSMCs. Levels are reported in pg/mL and was determined using a standard curve.

III.4 Discussion

ADSCs are increasingly being recognized for their highly potent capacity for differentiation. While it was originally postulated soon after their discovery in 2001 that they would be limited to osteogenic, chondrogenic and myogenic lineages, it is becoming increasingly clear that they have much wider applications. Due to the method of isolation and culture of ADSCs, the exact nature and origin of these cells is unclear and indeed it is unlikely that ADSCs are one cell type. In fact, what we refer to as ADSCs are actually the adherent population of the stromal vascular fraction. Some work has been done to characterize these cells, however considerable disagreement exists as to the immunophenotypic signature of these cells (see **Table 2.7**). As the use of these cells becomes more prevalent, considerable work will need to be done to fully characterize the

nature of the SVF and the exact identity of the cells that give rise to any cell differentiated from SVF. To this end, time lapse microscopy experiments will need to be performed to determine how MCs are produced in culture from ADSCs, in particular whether MCs bud off of adherent cells or if the adherent cells themselves release from the surface of the flask and become MCs. It was not in the scope of the present work to perform this characterization. ADSCs from bulk resected adipose tissue and liposuction aspirate collected from adult humans were successfully isolated and cultured. ADSCs isolated in this study showed a morphology and an immunophenotype largely consistent with those previously reported. One discrepancy between the immunophenotype determined for the ADSCs used in this study and some previously reported phenotypes was the lack of expression of CD105. As summarized in **Table 2.7** there is some disagreement about the use of CD105 as a marker of ADSCs as there are studies that show both expression and lack of expression of CD105.

As previously mentioned, there may be a bias induced by the nature of donors used in this study, namely that they were all female and many of whom were likely obese. ADSCs obtained from obese patients are likely to have biologically distinct functions and behaviors to those derived from donors with a normal body mass index. Obese adipose tissue is known to be more fibrotic than that of non-obese individuals [194] and connections between ADSC function and obesity in humans have been reported [195]–[197], including development of glucocorticoid resistance. The impact of these differences on the current study, however, is unknown and is unfortunately outside its scope. It would be valuable to determine if the health of the donor positively or

negatively affects the differentiative capacity of the cells produced as this may have implications for future therapies.

It has been shown here for the first time that functional human MCs can be produced through differentiation of ADSCs. ADMC are morphologically, phenotypically and functionally similar to other types of MCs. This was determined by direct comparison with hSMCs and correlation with previously reported observations in the literature.

The functional and morphological phenotypes of the ADMCs tested were found to be somewhat heterogeneous. The exact cause of this heterogeneity is unknown; however, it is likely comprised of several factors. It may be due to the more mature nature of the hSMCs used for this study. Cultures of hSMCs are not used for experiments until several months after their initial isolation, whereas ADMCs are used as they become available. It has been previously observed (unpublished observation and personal correspondence) that freshly isolated MCs tend to show higher levels of spontaneous release of granule contents. Another possible explanation for the differences in spontaneous release is that MCs derived from different tissues are phenotypically distinct and are very likely to be functionally distinct as well. This supports the hypothesized heterogeneity of ADMC cultures. It remains to be seen how this will affect their utility in future applications. It should be noted, however, that most types of cultured MCs show some level of heterogeneity. Furthermore, MCs in the body cannot be thought of as a monolithic population, with different tissue-originating MCs showing high degrees of variability in terms of the amount of each protease present in their granules.

In conclusion, MCs have been successfully differentiated from stem cells isolated from human adipose tissue via a yet to be determined mechanism. These cells are phenotypically, functionally and genetically similar to mature tissue derived MCs isolated from skin.

CHAPTER IV

APPLICATION OF ADIPOSE DERIVED MAST CELLS TO BREAST CANCER AND ALLERGO-ONCOLOGY

IV.1 Introduction

Breast cancer is the most diagnosed malignancy and the second cause of cancer death in women in the United States. The prognosis for breast cancer patients is particularly poor in those bearing tumors overexpressing HER2/*neu*. While treatment of patients with advanced breast cancer using the FDA anti-HER2/*neu* IgG1 Ab (Ab) trastuzumab (Herceptin®) leads to improvement in some patients with HER2/*neu* positive tumors, most acquire resistance and eventually die. Thus, improved therapies for HER2/neu expressing breast tumors are still urgently needed. In general, Abs used in the clinic against cancer are of the IgG class. IgE Abs have several properties that make them attractive for cancer therapy such as is its extremely high affinity for its Fc epsilon receptor I (FcεRI), making it a cytophilic Ab stably bound to the surface of effector cells expressing the receptor, such as the tissue-based immune cells MCs (MC). Previous studies have shown that high MC densities in breast tumors are associated with favorable prognoses suggesting a protective role. Importantly, MC release (upon FcεRI stimulation) relevant anti-cancer agents such as tumor necrosis factor alpha (TNF-α), uniquely stored in human MC granules, as well as granulocyte macrophage colony-stimulating factor (GM-CSF). Thus, discovering approaches to target autologous MC to tumor antigens is

an unrecognized form of a potential particularly effective cancer immunotherapy. However, this concept has not been realized given that MC reside in tissue; thus, efficient removal, expansion *ex vivo*, and targeting to tumor sites is not yet feasible. In addition, protocols aimed to differentiate MC *in vitro* using cord blood or peripheral blood as a progenitor source are time consuming and limited by the fact that MC maturation cannot be fully achieved.

The overall goal of this project is to investigate a novel autologous, MC-based cancer immunotherapy to treat breast cancer. We discovered that human, autologous fully differentiated MC can be obtained from adipose tissue and successfully expanded *ex vivo*. These ADMCs, armed with IgE anti-HER2/*neu*, are activated by HER2/*neu* human breast cancer cells to release GM-CSF and TNF-α, bind to and kill breast cancer cells, and are not activated by monomeric (shed) HER2/*neu* (found in HER2/*neu* breast cancer patient serum). Our discovery could potentially lead to completely new and innovative ways to treat malignancies by harnessing naturally occurring anti-tumor agents within autologous (or allogeneic) MC.

We hypothesized that ADMC effectively armed with anti-HER2/*neu* IgE Abs would target breast cancer cells, degranulate upon antigen (Ag) exposure, and kill the breast cancer cells *in vitro* and *in vivo*. Their interaction with the high density of HER2/*neu* Ag expressed on the surface of malignant cells would create a local IgE-targeted immediate hypersensitivity (anaphylactic) reaction, resulting in acute inflammation in the tumor microenvironment and in the rapid killing of cancer cells. Further benefit of this strategy is anticipated as the presence of dead tumor cells would

allow the effective uptake and presentation of tumor antigens by antigen-presenting cells, such as dendritic cells resulting in efficient priming of an adaptive broad-spectrum cellular immune response not only to HER2/*neu* but also to other tumor antigens due to epitope spreading. Here, it is shown this strategy is effective *in vitro* as the ADMC, armed with tumor targeting IgE, selectively bind to and kill breast cancer cells upon FcεRI stimulation.

IV.2 Methods

IV.2.i SK-BR-3

The HER2/*neu* expressing human breast adenocarcinoma cell line, SK-BR-3 was purchased from the American Type Culture Collection and cultured in McCoy's 5A Medium supplemented with 10% FBS and 1% Pen/Strep in T-25 flasks in a 37°C incubator with a 5% CO_2 atmosphere. Media changes were performed every two to three days. Flasks were passaged by removing cells from the flask surface using CellStripper™ Cell Dissociation Reagent, pelleting the cells then re-seeding a flask with one fifth the original number of cells. Passages were performed when cells reached approximately 90% confluency.

IV.2.ii Cytospins

In order to perform immunohistochemistry on cells that are weakly or non-adherent it is necessary to first immobilize them on a glass substrate. This is most commonly achieved using a cytospin apparatus which uses centrifugal force to deposit cells onto glass microscope slides. Once attached, slides are briefly air dried, fixed with methanol, and incubated with Abs specific for either activated caspase or tryptase (5μg/mL) overnight in a humidified chamber kept at 4°C in the dark. Following primary Ab staining, slides were washed to remove any unbound primary Ab. Secondary Ab staining was performed for 2 hours also at 4°C in the dark in a humidified chamber, after which slides were washed and mounted under coverslips and allowed to cure overnight. Images were collected using a Zeiss spinning disc confocal microscope.

IV.2.iii ADMC Co-Culture with Breast Cancer Cells - ADCC

Initial experiments were performed using two cell stains, MitoTracker™ Red to stain SK-BR-3 cells and MitoTracker™ Green to stain ADMC. These experiments lasted six hours each. In order to incorporate propidium iodide staining into the protocol, in subsequent experiments CellTracker Deep Red was used to stain ADMC and MitoTracker™ Green was used to stain SK-BR-3 cells and experiments lasted between 48 and 72 hours. For co-culture experiments, ADMCs were collected, pelleted then resuspended in X-Vivo supplemented with rhSCF but without IgE and plated in a 24 well cell culture plate. ADMCs were maintained in media without IgE for a minimum of 4 days to allow IgE bound to FcεRI molecules on the cell surface to be released. This step

was added to ensure that the receptors were not saturated with IgE upon addition of the Abs being investigated. SK-BR-3 cells were removed from flasks as described above. Cells were seeded into 60 mm plastic petri dishes with cover slip bottoms at a density of either 50,000 or 160,000 cells/3 mL, depending on the experiment. SK-BR-3 cells were allowed to attach and grow for 24 hours. After at least four days of incubation, 160,000 ADMCs per condition were collected from the non-IgE media were incubated with either 1 μg/mL anti-HER2 or control IgE. ADMC were incubated with 2 μM CellTracker Deep Red or MitoTracker™ Green for one hour on ice. Media was removed from SK-BR-3 cells and replaced with fresh media containing 2 μM MitoTracker™ Green or Red for 30 minutes at 37°C in the dark. Following 30 minutes of staining, SK-BR-3 cells were washed once with fresh media to remove unincorporated dye and 1 mL of fresh media was added to the plate. Following staining and activation with IgE, ADMC were pelleted and washed once to remove unincorporated dye and unbound IgE, then resuspended in 1 mL of SK-BR-3 media and added to the plate containing SK-BR-3 cells. A final 1 mL of SK-BR-3 media containing Propidium Iodide (in some experiments) is added to the plate to yield a final concentration of 25 μg/mL. Imaging was begun as soon as possible, usually five minutes, but not exceeding 20 minutes. Images were collected using a Zeiss spinning disc confocal microscope fitted with an environmental incubation chamber designed to maintain an environment of 37°C and 5% CO_2. MitoTracker™ Green was imaged using a 488 nm laser at 2% intensity, propidium iodide was imaged using a 561 nm laser at 3% intensity and CellTracker Deep Red was imaged using a 639 nm laser at

10% intensity. For each time point and, in each color, images were taken from 10 focal planes with a spacing of 12.73 μm.

IV.2.iv ADMC Mediator Induced Cell Killing (MICK)

SK-BR-3 cell death induced by mediators released from stimulated ADMCs was measured using a similar protocol as described for ADMC-ADCC. Mediator release from at least one million ADMCs was induced as described for the β-hexosaminidase release assay. Briefly, ADMC were collected, counted and pelleted by centrifugation. Cells were then incubated with 1 μg/mL of the FcεRI Ab (3B4) overnight and allowed to release preformed as well as newly generated mediators. Following incubation, cells were pelleted, and supernatants were removed. Supernatants were filtered through 0.22 μm filters to ensure that no cells were present in mediator preparations. SK-BR-3 cells were seeded into 60 mm cover slip bottom petri dishes at a density of 160,000/3 mL and allowed to attach and grow for 24 hours before use. On the day of the experiment media was removed from cells and replaced with fresh media containing 2 μM MitoTracker™ Green and incubated at 37°C in the dark for 30 minutes. Following staining, cells were washed once to remove unincorporated dye and replated in 3 mL of fresh media containing 25 μg/mL of propidium iodide and between 500 and 1000 μL of mediator preparations. Images were collected as described above with the exception of the 639 nm laser which was not used.

IV.2.v Extended Time Course Experiments

For extended time course experiments lasting more than 72 hours, SK-BR-3 cells were prepared similarly to previous experiments with the exception of the omission of any cell-labeling dyes. Propidium iodide was added to the media as above and images were collected at the beginning of each experiment and at various timepoints thereafter. Unlike previous experiments in which cells were maintained on the microscope in an environmental control chamber, for extended time course experiments the plates were kept in a separate incubator and removed for imaging.

IV.3 Results

IV.3.i ADMC Co-Culture with Breast Cancer Cells

ADMC co-culture experiments were performed to investigate the ability of MCs targeted through Trastuzumab IgE to induce cytotoxicity in SK-BR-3 cells. In the first co-culture experiments, ADMC were stained with MitoTracker™ Green and sensitized with either Trastuzumab IgE or non-specific psIgE and SK-BR-3 cells were stained with MitoTracker™ Red, and observed over the course of six hours, as seen in representative images from several time points in **Figure 4.1.** During this time, interactions were observed in cells incubated with both specific and non-specific Abs. As seen in **Figure 4.2**, dye was often observed to transfer from the breast cancer cells into ADMC, however dye was not regularly observed to pass from MCs into breast cancer cells. In experiments using Trastuzumab IgE, ADMC were observed to translocate considerable distances toward breast cancer cells (**Figure 4.3**), whereas this translocation was not observed

during the six-hour experiments with non-specific IgE. Another phenomenon only observed in the trastuzumab IgE experiment was the induction of polarization of ADMC staining, possibly indicative of MC activation through IgE-bound FcεRI binding to HER2/*neu* expressing breast cancer cells (**Figure 4.4**).

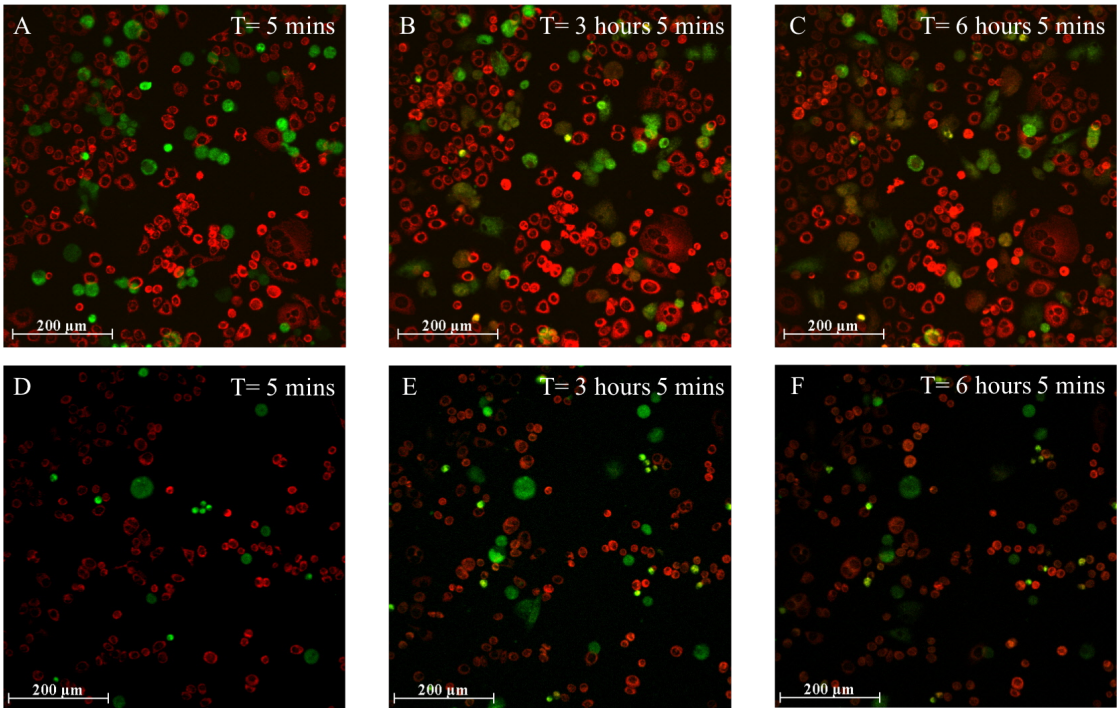

Figure 4.1. Six-Hour ADMC and SK-BR-3 Co-Culture. 259,000 ADMC were sensitized with 1 µg/ml of a non-specific control IgE (A-C) or trastuzumab IgE (D-F) and stained with MitoTracker™ Green. The MitoTracker™ Green-loaded ADMC were added to adherent SK-BR-3 (10^5-10^6) that had been pre-stained with MitoTracker™ Red and time lapse video taken every five minutes over six hours at 10x magnification.

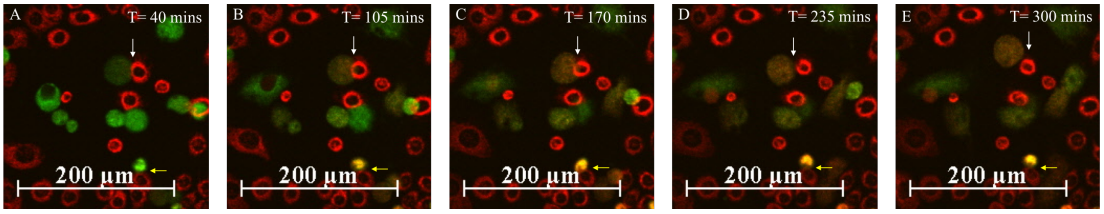

Figure 4.2. ADMC-to-SK-BR-3 Dye Transfer. ADMCs were sensitized with 1 µg/mL non-specific IgE for 1 hour and stained with MitoTracker™ Green. SK-BR-3 cells were stained with MitoTracker™ Red. White and yellow arrows indicate ADMC-SK-BR-3 interactions. ADMCs are seen to uptake MitoTracker™ Red from SK-BR-3 cells. Images were collected every five minutes for six hours at a magnification of 10x.

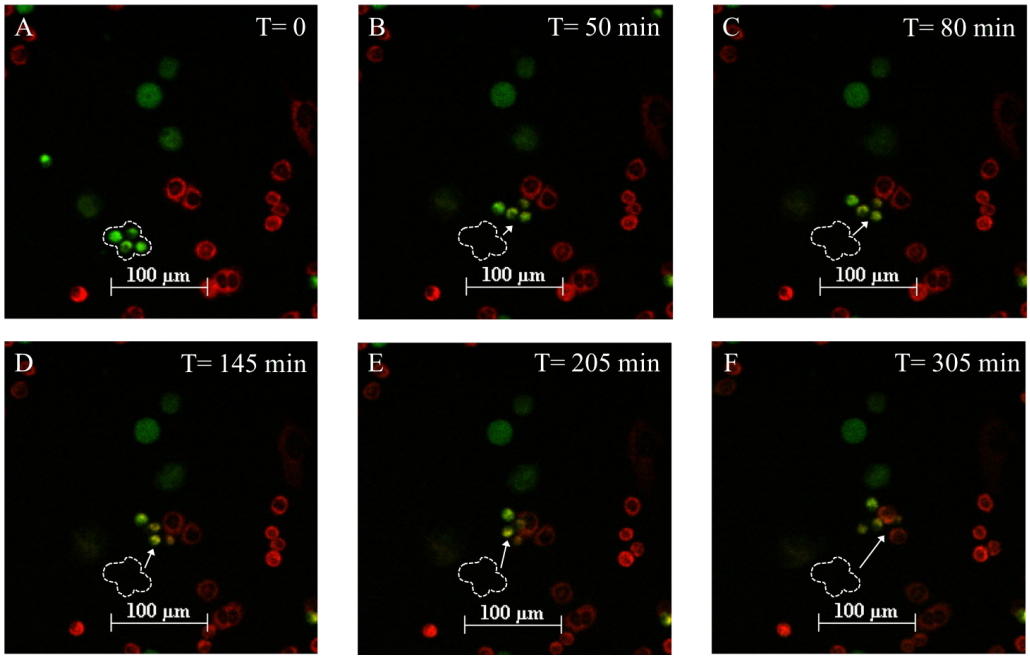

Figure 4.3. ADMC Binding of SK-BR-3 Cells. 259,000 ADMC were sensitized with 1 µg/ml of trastuzumab IgE and stained with MitoTracker™ Green. The MitoTracker™ Green-loaded ADMC were added to adherent SK-BR-3 (10^5-10^6) that had been pre-stained with MitoTracker™ Red and time lapse video taken over six hours. The white circular boundaries and arrows represent starting point and tracking of ADMC (green) at time 0 to SK-BR-3 (red) binding over five of the six hours at 10X magnification.

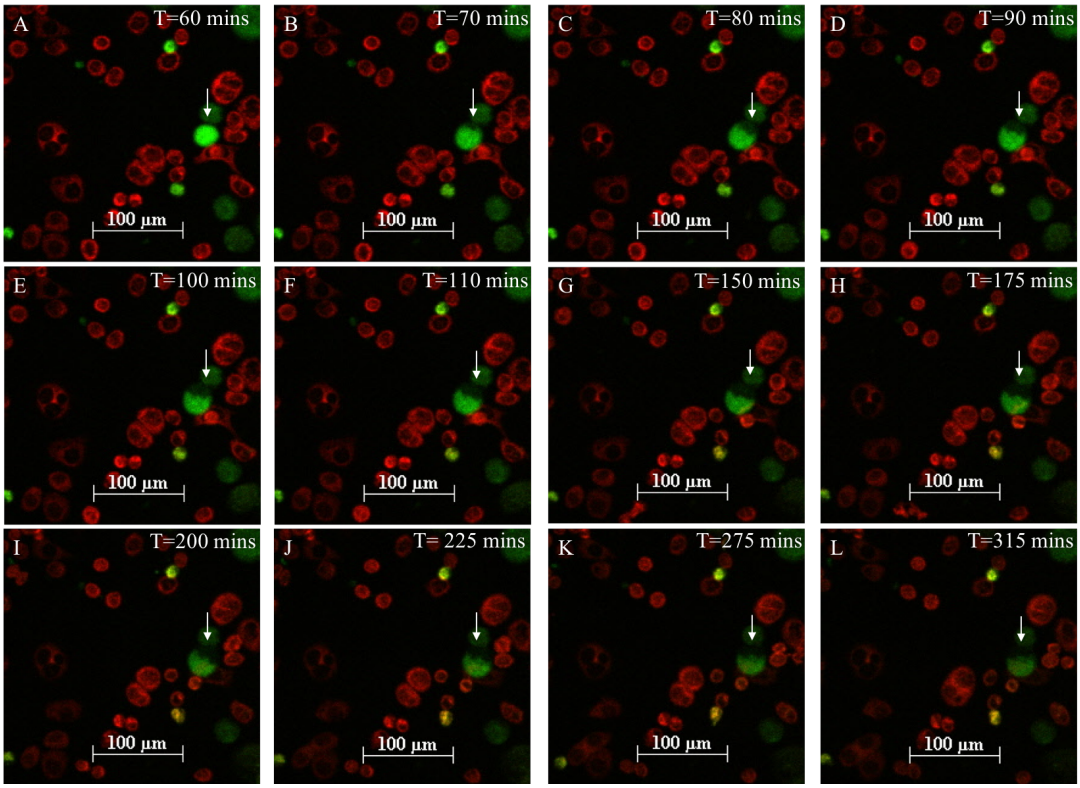

Figure 4.4. Cytoplasmic Polarization of ADMC. ADMCs were sensitized with 1 µg/mL Trastuzumab IgE for 1 hour and stained with MitoTracker™ Green. SK-BR-3 cells were stained with MitoTracker™ Red. White arrow shows ADMC induced to polarize cytoplasm towards SK-BR-3 cell. Images were collected every five minutes over the course of six hours at 10x magnification.

The initial protocol for monitoring SK-BR-3 cell killing by ADMC used ADMC labeled with MitoTracker™ Green, unlabeled SK-BR-3 and propidium iodide in the media and co-cultures were monitored over the course of 18 hours. As seen in **Figure 4.5**, over the course of the experiment the dye was observed to stain the cancer cells that were initially unstained. The problem of dye leakage prompted the further augmentation of the initial protocol to a more cell-permanent dye, CellTracker™ Deep Red. As can be

seen in **Figures 4.6**, using this system, dye leakage was eliminated, and it became possible to increase experiment durations to up to 72 hours.

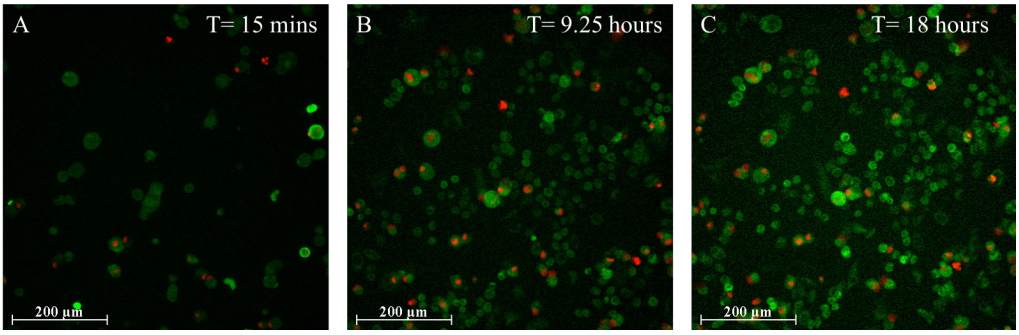

Figure 4.5. MitoTracker™ Green Leakage from ADMC. Roughly 420,000 ADMCs were sensitized with 1 µg/mL Trastuzumab IgE for 1 hour and stained with MitoTracker™ Green. Images were collected every 15 minutes for 18 hours at 10x magnification.

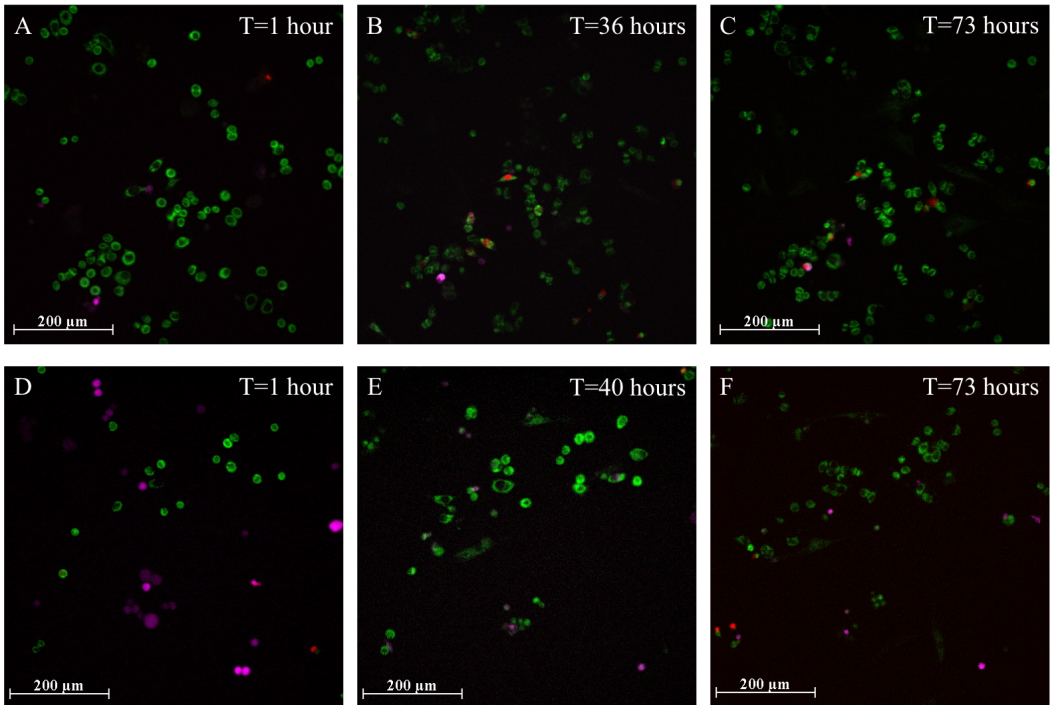

Figure 4.6. Three Color ADMC-SK-BR-3 Co-Culture Experiments. 160,000 ADMC were sensitized with Trastuzumab IgE (A-C) or non-specific IgE (D-F) at 1 µg/mL for one hour and stained with CellTracker™ Deep Red. 160,000 SK-BR-3 cells were stained with MitoTracker™ Green and propidium iodide was added to culture media. Images were collected every hour for 72 hours at a magnification of 10x.

ADMC sensitized with Trastuzumab IgE induced PI uptake in cancer cells, however PI uptake was also induced by MCs sensitized with control IgE. In at least one instance in which MCs were co-cultured with breast cancer cells for long periods, a MC was observed to phagocytose a cancer cell (**Figure 4.7**). Cancer cell phagocytosis was an uncommon occurrence that was observed in experiments using Trastuzumab IgE and psIgE.

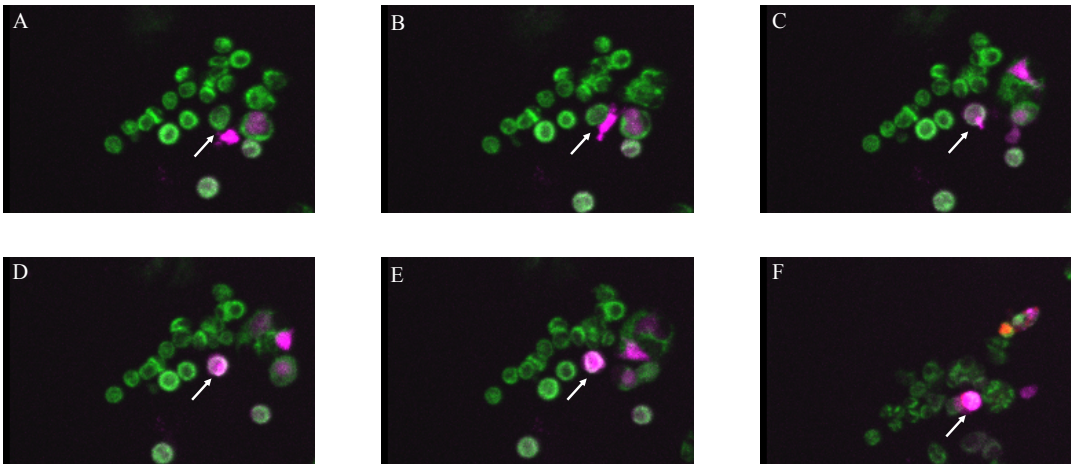

Figure 4.7 Phagocytosis of SK-BR-3 Cell. Roughly 140,000 ADMCs were sensitized with a non-specific IgE for one hour and stained with CellTracker™ Deep Red. 160,000 SK-BR-3 cells were stained with MitoTracker™ Green. Selected images show phagocytosis of SK-BR-3 cell by an ADMC. White arrow indicates cells of interest. Images were collected every hour for 72 hours at 10x magnification.

IgE sensitized ADMCs were co-incubated with SK-BR-3 cells overnight then collected and used to make cytospin preparations that were then probed with Abs specific for activated caspase and tryptase. As seen in **Figure 4.8**, IgE-sensitized ADMC induced a significant level of caspase activity. **Figure 4.9** shows that caspase activation was almost non-existent in non-stimulated-ADMC co-cultures with SK-BR-3 cells.

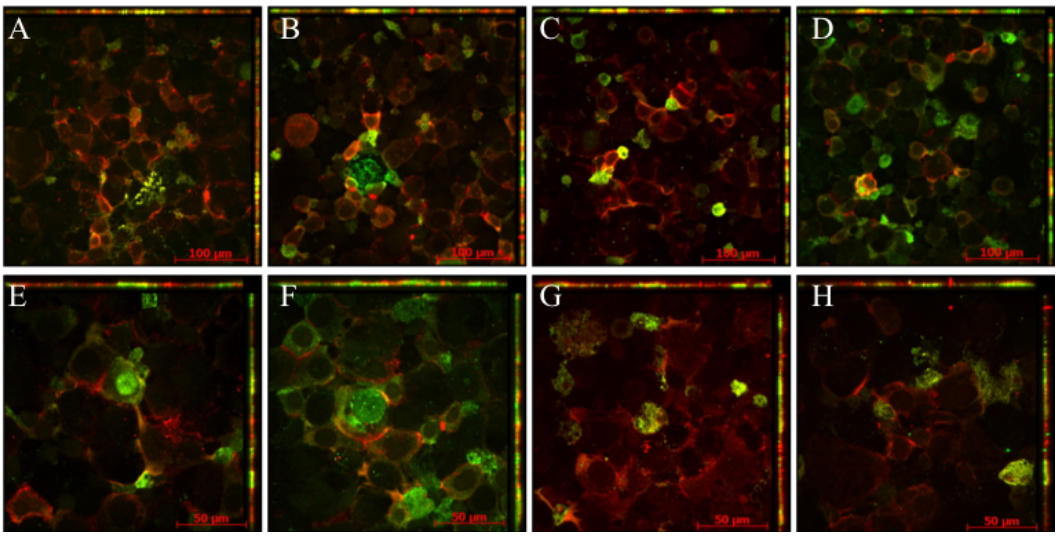

Figure 4.8. Induction of Apoptosis in SK-BR-3-ADMC Co-Cultures. Trastuzumab IgE-sensitized ADMC (7.5x104) were incubated with SK-BR-3 ($1x10^5$) for 72 hours, cytospins made, fixed, and incubated with Alexa Fluor™ 488 labelled, mouse anti-human tryptase (green) along with Alexa Fluor™ 647 labelled, mouse anti-human caspase 3 (red). A-D were collected using 20x magnification, E-H were collected using 40x magnification.

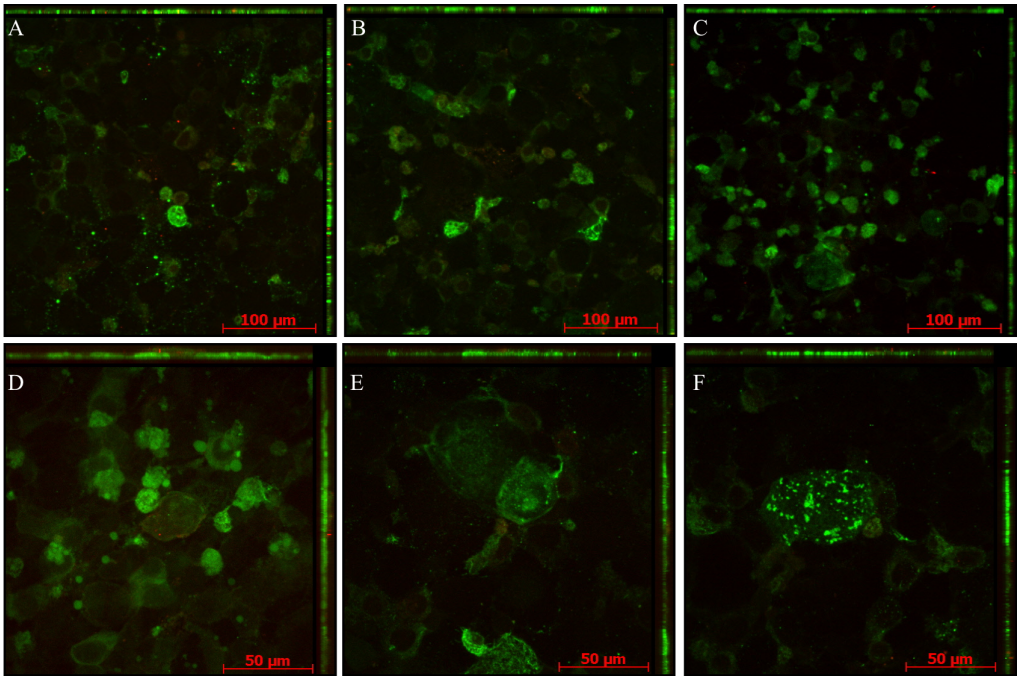

Figure 4.9. Non-Specific IgG Controls. Trastuzumab IgE-sensitized ADMC ($7.5x10^4$) were incubated with SK-BR-3 ($1x10^5$) for 72 hours, cytospins made, fixed, and incubated with Alexa Fluor™ 488 labelled, mouse anti-human tryptase (green) along with Alexa Fluor™ 647 labelled, isotype control IgG for caspase 3 Ab (red). A-D were collected using 20x magnification, E-H were collected using 40x magnification.

IV.3.ii ADMC Mediator Induced Cell Killing

To further explore the mechanism of ADMC induced cytotoxicity in SK-BR-3 cells, the participation of cells as opposed to substances released from cells was examined. ADMCs were stimulated with the FcεRI crosslinking Ab 3B4 overnight and supernatants were collected and added to dishes containing SK-BR-3 cells in the presence of propidium iodide. As seen in **Figure 4.10**, mediators from FcεRI-challenged MCs induced low levels of apoptosis as determined by propidium iodide uptake at successive time points. Apoptosis determined through activated caspase staining showed higher

levels of apoptosis in SK-BR-3 cells treated with FcεRI-challenged MC supernatants (**Figure 4.11**) than in cells treated with non-challenged ADMC supernatants (**Figure 4.12**).

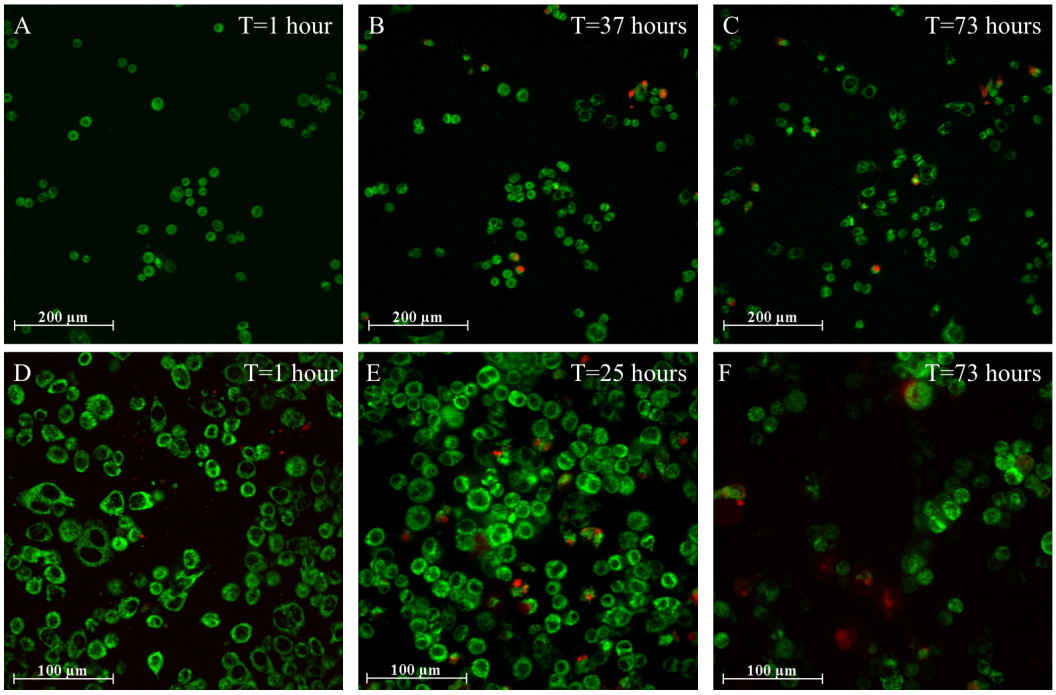

Figure 4.10. ADMC-MICK of SK-BR-3 Cells. 160,000 SK-BR-3 cells were stained with MitoTracker™ Green for 30 minutes. Propidium iodide was added to the culture media in addition to roughly 500 µL of FcεRI-challenged MC supernatant. Images were collected once every hour (A-C) or every 30 minutes (D-F) for 72 hours at a magnification of 10x (A-C) or 20 x (D-F).

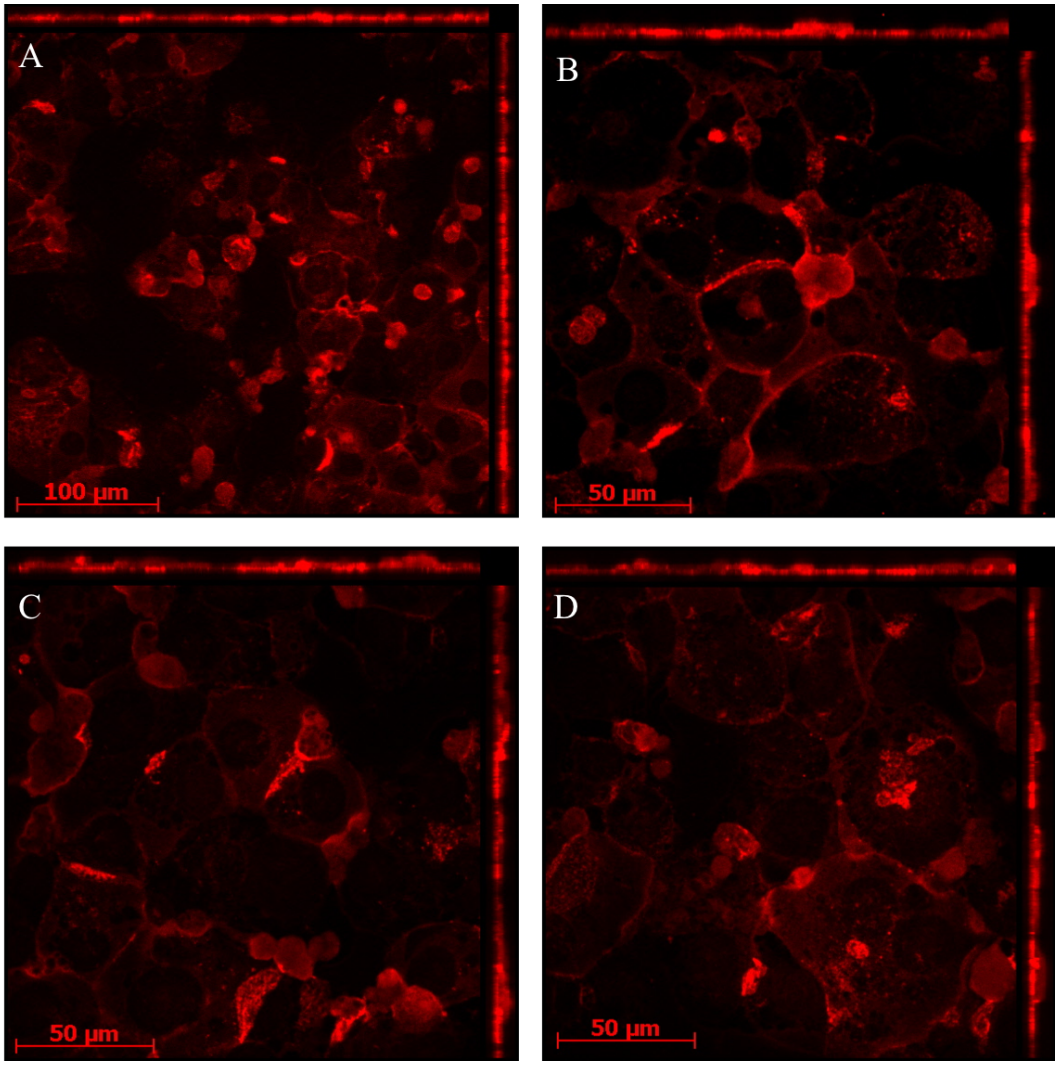

Figure 4.11. Induction of Apoptosis in SK-BR-3 by Activated MC Mediators. ADMC ($1.3x10^6$) were challenged with optimal concentrations of anti-FcεRI stimuli (70% release) for 24 hours and supernatants (no cells) from these ADMC were incubated with SK-BR-3 cells (10^5) for 72 hours, cytospins prepared, fixed, and incubated with Alexa Fluor™ 647 labelled, anti-human caspase 3 (red). Images were collected at 20x (A) and 40x (B-D).

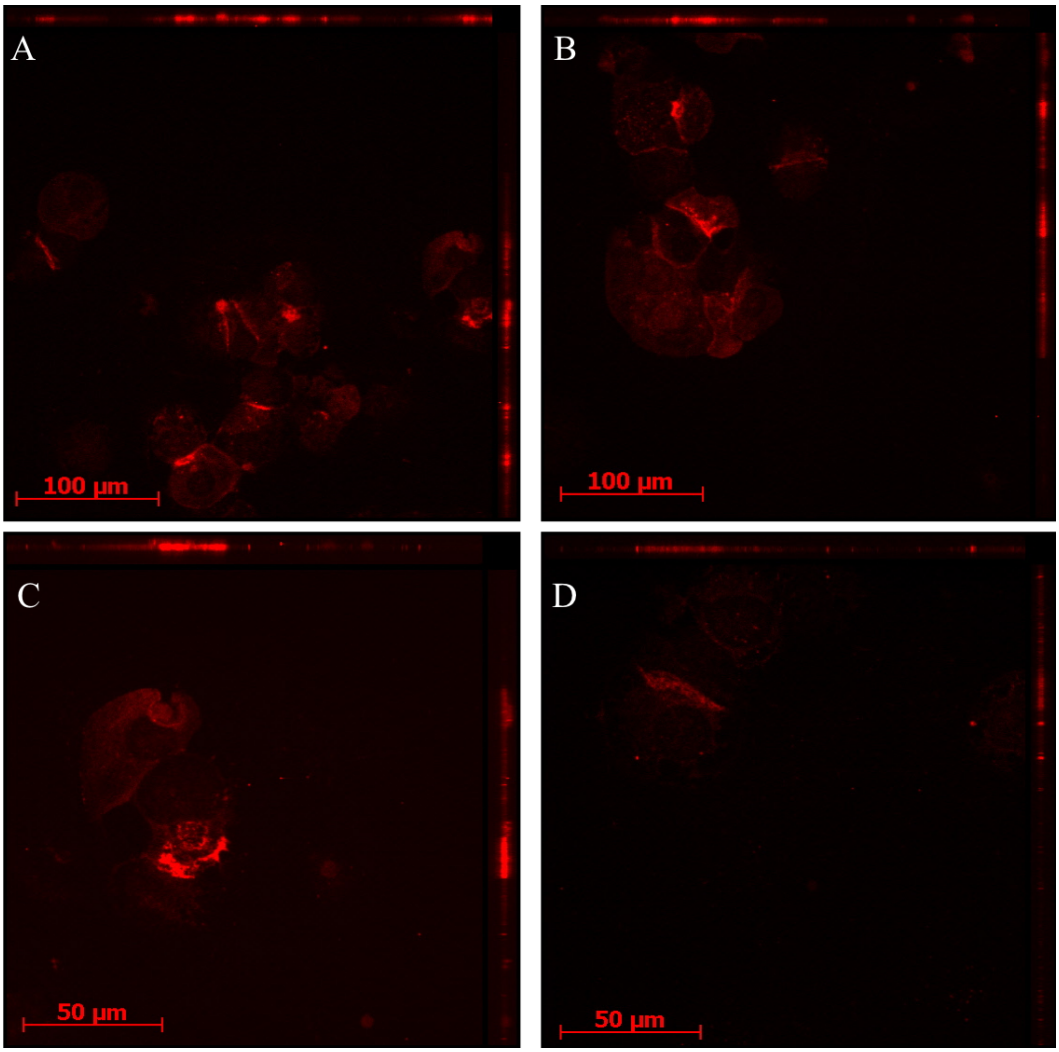

Figure 4.12. Induction of Apoptosis in SK-BR-3 by Non-Activated MC Supernatants. Media (no cells) from non-FcεRI challenged ADMC were incubated with SK-BR-3 (10^5) for 72 hours, cytospins prepared, fixed, and incubated with Alexa Fluor™ 647 labelled, anti-human caspase 3 (red). Images were collected at 20x (A-B) and 40x (C-D).

IV.3.iii 72+ Hour Experiments

In an effort to more accurately quantify the uptake of PI over longer time spans, a protocol was created wherein cells were grown in an incubator and removed at various time points for imaging. Cells were grown for 96 hours in the presence of supernatants from FcεRI-challenged or non-challenged MCs and images were collected over the course of the experiment. Supernatants from FcεRI-challenged MCs induced a loss of membrane integrity in breast cancer cells at a higher level than did control supernatants.

During the first experiment, both ADCC and MICK were investigated. Measurements were taken at day 0, day 5 and day 7 and as illustrated in **Figure 4.13**, although the treated cells showed higher levels of PI uptake, both control samples also showed a statistically significantly higher level of PI uptake at day 7 vs day 5.

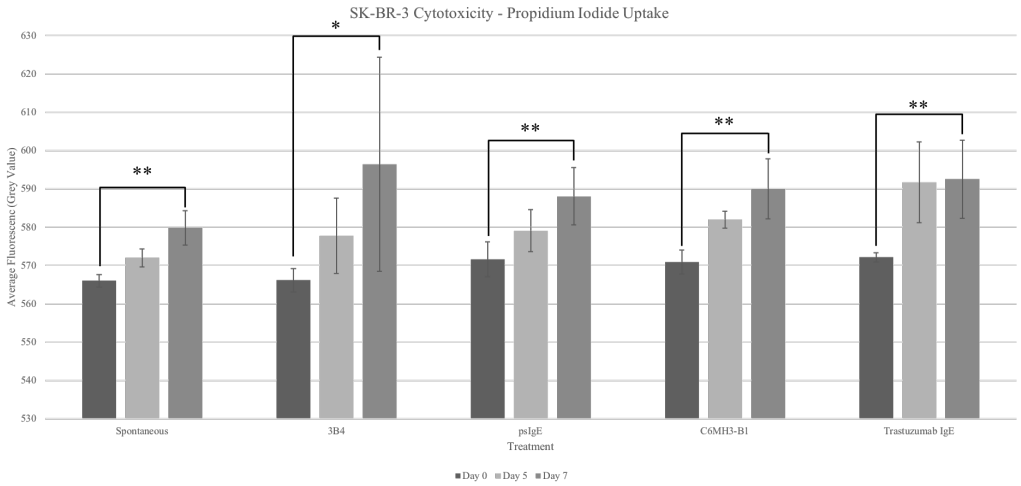

Figure 4.13. Quantification of Cell Membrane Integrity Loss Over Seven Days. 160,000 SK-BR-3 cells were cultured in glass-bottom dishes with propidium iodide and either supernatant (spontaneous, 3B4) or ADMC sensitized with an IgE. Dishes were imaged at four locations for every time point and averages were calculated (* denotes P < 0.05, ** denotes P < 0.005, ns denotes P > 0.05). P values calculated using type 2, 2 tailed students t-test.

In the next experiment ADCC was measured over the course of 14 days. As can be seen in **Figure 4.14**, the trend observed after 7 days continued to day 14.

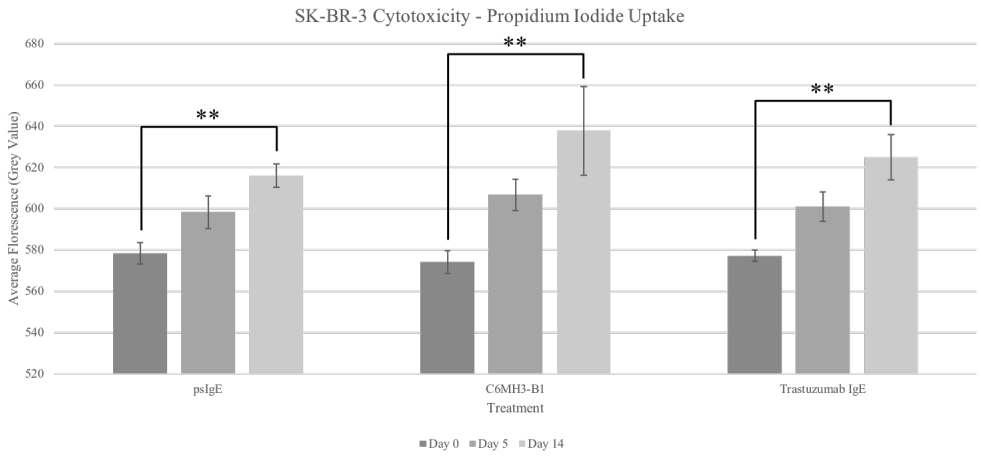

Figure 4.14. Quantification of Cell Membrane Integrity Loss Over 14 Days. 160,000 SK-BR-3 cells were cultured in glass-bottom dishes with propidium iodide and ADMC sensitized with an IgE. Dishes were imaged at four locations for every time point and averages were calculated (* denotes P < 0.05, ** denotes P < 0.005, ns denotes P > 0.05). P values calculated using type 2, 2 tailed students t-test.

In the third experiment the contribution of non-sensitized MCs was investigated and compared to ADCC over the course of four days. As can be seen in **Figure 4.15**, after four days non-sensitized MCs induced a small but statistically significant increase in PI uptake.

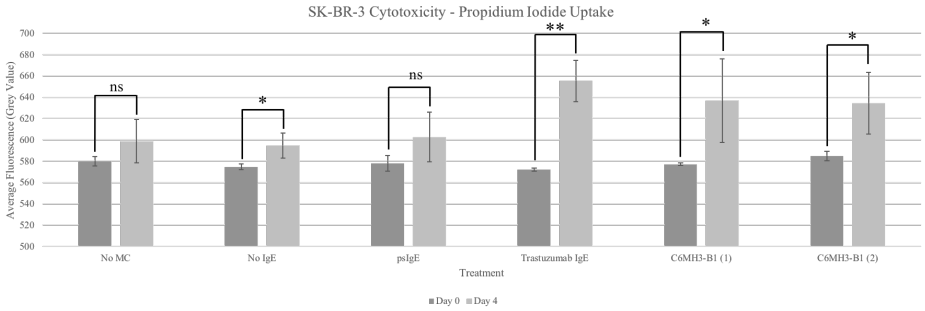

Figure 4.15. Quantification of Cell Membrane Integrity Loss Over Four Days. 160,000 SK-BR-3 cells were cultured in glass-bottom dishes with propidium iodide alone (no MC), supernatant (spontaneous, 3B4), non-sensitized ADMC (no IgE) or ADMC sensitized with an IgE. Dishes were imaged at four locations for every time point and averages were calculated (* denotes $P < 0.05$, ** denotes $P < 0.005$, ns denotes $P > 0.05$). P values calculated using type 2, 2 tailed students t-test.

In the final experiment, MICK was investigated over the course of four days. As can be seen in **Figure 4.16**, FcεRI-challenged MC supernatants induced a significantly significant increase in PI uptake after four days, whereas neither control condition did.

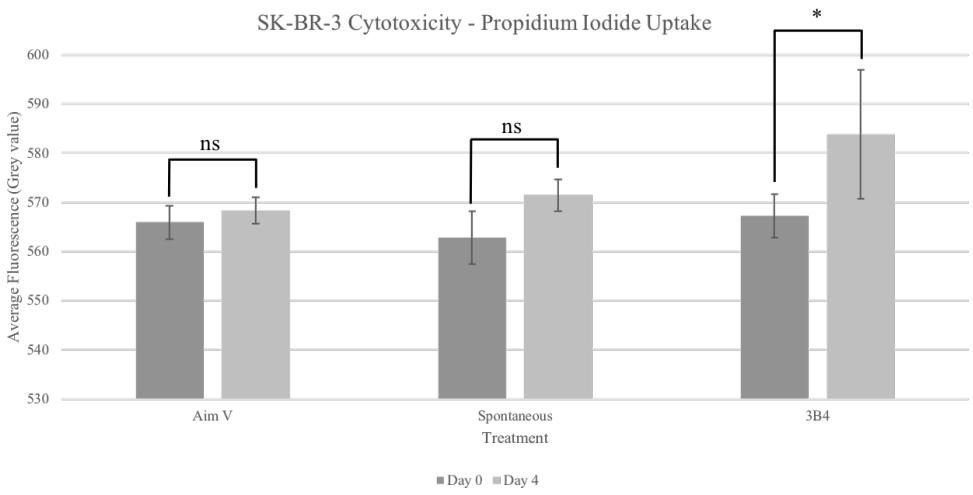

Figure 4.16. Quantification of Cell Membrane Integrity Loss Over Four Days. 160,000 SK-BR-3 cells were cultured in glass-bottom dishes with propidium iodide and either fresh media (AimV), supernatant from non-stimulated ADMC (spontaneous), or FcεRI-challenged ADMC (3B4). Dishes were imaged at four locations for every time point and averages were calculated (* denotes $P < 0.05$, ** denotes $P < 0.005$, ns denotes $P > 0.05$). P values calculated using type 2, 2 tailed students t-test.

IV.4 Discussion

ADMC induced cytotoxicity was observed in co-cultures of MCs and breast cancer cells with a large degree of heterogeneity. Cell death was most commonly observed after roughly 96 hours, although interactions between MCs and breast cancer cells were observed earlier very soon after the introduction of MCs to the dish. Cell-cell interactions appeared to be partly Ab mediated and partially independently of Ab specificity as control IgE-laden MC were also observed to interact with breast cancer cells.

The first set of experiments were performed over the course of six hours, during which time interactions were observed between ADMCs and SK-BR-3 cells regardless of

the Ab used to activate the ADMC. However, considerably more interactions were observed when anti-HER2 IgE was used. In addition to interactions between cells such as localization or dye transfer, in at least one instance a polarization of cytoplasmic contents of a MC towards a cancer cell was observed. It is not clear if this represents something akin to an immunological synapse [198], a degranulatory synapse [199], or something else altogether. No specific staining was used that would confirm or deny the presence of molecules indicative of a synapse as this was not feasible using the live-cell imaging protocol used for these studies. Future experiments in which synapse formation is investigated should be undertaken.

Another interesting phenomenon that was directly observed at least once was phagocytosis of a cancer cell by a MC. MC phagocytosis of breast cancer cells *in vivo* has been suggested, and there is circumstantial evidence supporting this [200]. The degree to which cancer cell phagocytosis by MCs represents a physiologically [201] or therapeutically relevant process will require further investigation, however it is consistent with reported antigen presenting cell capabilities of MCs [202].

It is interesting to note that MCs seem to have a significant level of basal cancer cell cytotoxicity as evidenced by the induction of cell killing by supernatants from non-FcεRI-challenged MCs, non-sensitized ADMC or ADMC sensitized with non-specific IgE. This is in contrast to media alone which failed to induce statistically significant levels of cell death. It is not clear what is responsible for this observation, and further research will be necessary to determine if the mechanism of cytotoxicity is the same or different from those responsible for ADCC and MICK.

One shortcoming of these initial experiments was the lack a condition with no IgE. While a control IgE, psIgE was used, the exact nature of this control IgE is unknown. Originally collected from a patient with an IgE-overproducing malignancy, this IgE has not to the best of the knowledge of the author been characterized for specificity or affinity of binding. Given this uncertainty it would be of interest to examine a second non-specific IgE, as the possibility exists that the interaction of monomeric IgE with FcεRI has a stimulatory effect on ADMC.

Another shortcoming of this experimental setup was the use of propidium iodide rather than a more specific marker of apoptosis. Propidium iodide is in fact a marker of cell membrane integrity loss, which is associated with cell death, but may also be influenced by other factors. It will be necessary to replicate these results in the future to address these concerns. Dyes that can be used in live-cell time lapse imaging do exist and were purchased, specifically CellEvent™ Green. Preliminary experiments suggest that it may be possible to replace propidium iodide with CellEvent™ Green. As shown in **Figure 4.17**, CellEvent™ Green staining often overlaps with PI staining, however some differences exist. It was not possible to incorporate its use into the current set of experiments; however, it will be interesting to investigate their utility in the future.

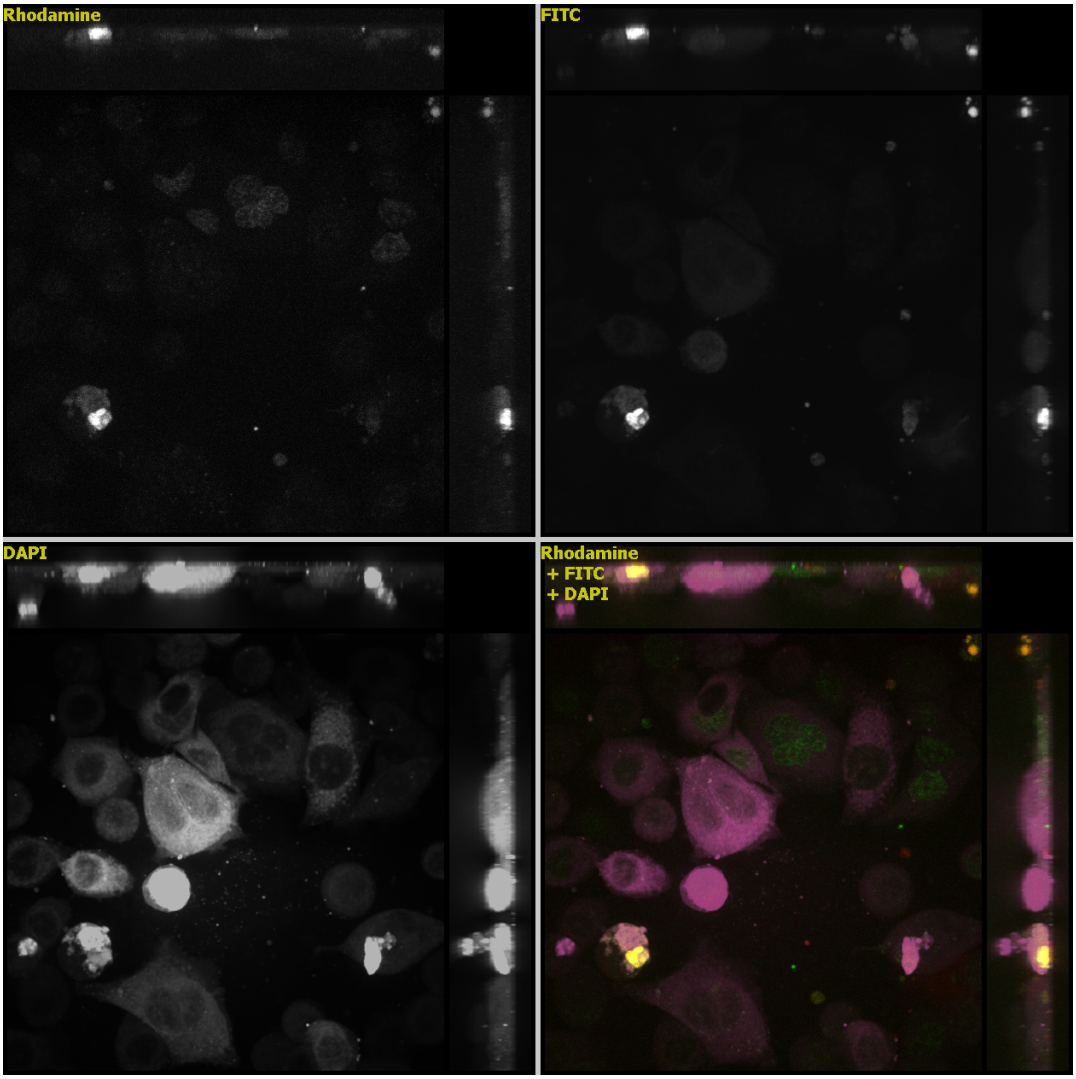

Figure 4.17. CellEvent™ Green Test. SK-BR-3 cells were stained with CellTracker™ Deep Red, and PI and CellEvent™ Green were added to the culture media. Cells were grown for 24 hours and images were taken at 40x magnification.

Another possible change to the current experimental setup would be the use of flow cytometry as opposed to confocal microscopy as a means to quantify staining by the apoptosis marker of choice. As with CellEvent™ Green, some preliminary experiments

were performed to test the feasibility of this method. As shown in **Figure 4.18**, two populations of cells could be differentiated based on PI staining.

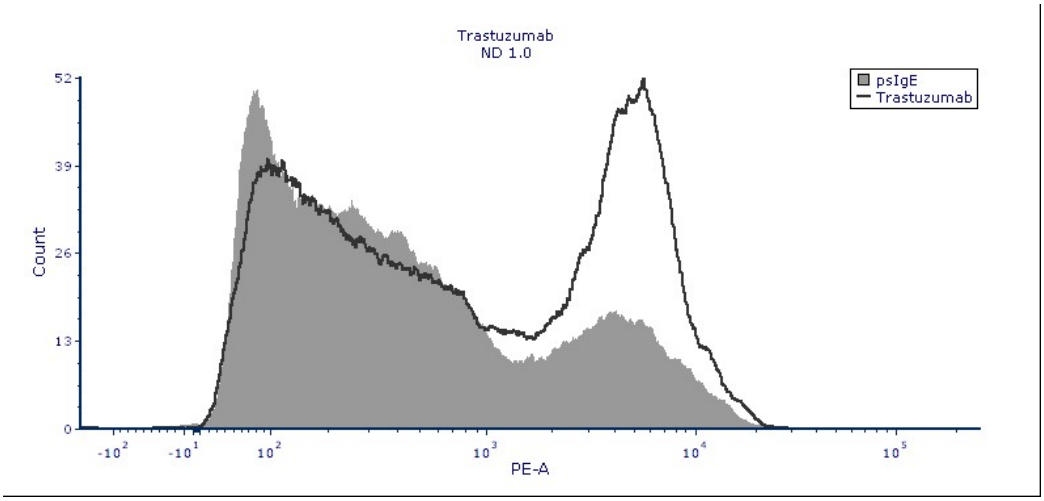

Figure 4.18. Flow Cytometric Analysis of ADCC Experiments – Trastuzumab-IgE. Following image collection at hour 96 of ADCC experiments, cells were collected using CellStripper™ and analyzed using a FACSAriaIII™ flow cytometer to measure PI fluorescence. Filled in gray curve represents non-specific IgE control, black line represents trastuzumab-IgE sensitized ADMC-SK-BR-3 co-cultures.

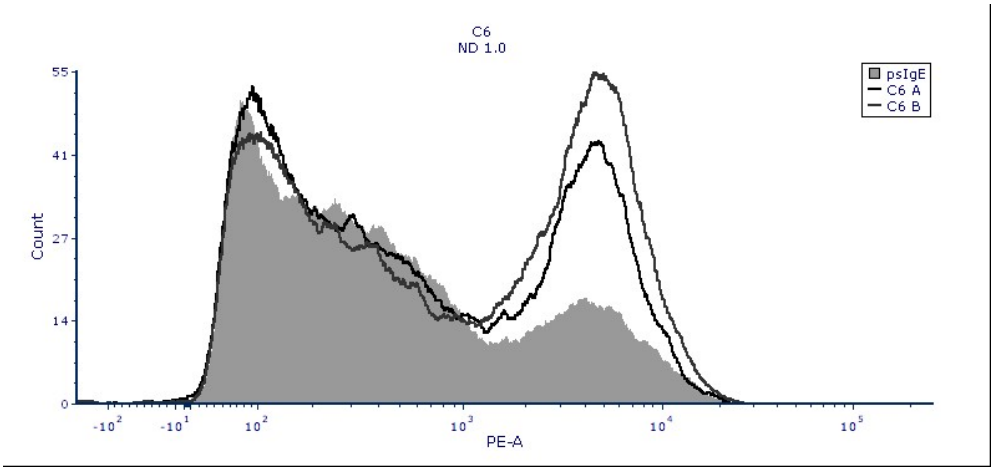

Figure 4.19. Flow Cytometric Analysis of ADCC Experiments – C6MH3-B1. Following image collection at hour 96 of ADCC experiments, cells were collected using CellStripper™ and analyzed using a FACSAriaIII™ flow cytometer to measure PI fluorescence. Filled in gray curve represents non-specific IgE control, black line and dark grey lines represent C6MH3-B1-IgE sensitized ADMC-SK-BR-3 co-cultures.

The exact role that MCs play in the progression, maintenance and spread of tumors in the human body remains largely unclear, due to the seemingly contradictory results of studies of the effect that MCs have on tumors. While this may be in part due to differences in experimental setups, it is also likely due to the highly pleiotropic nature of MCs and their ability to behave in many often starkly contrasting context-specific ways. It is likely that there are certain malignancies that would benefit from the increased presence of MCs while other others may be exacerbated by their presence. The current study focused on HER2 positive breast cancer as it is one of the types of tumors in which the presence of MCs is associated with higher survival rates. It remains to be seen if this type of therapy could be utilized in tumors not traditionally associated with MCs having a positive effect on survival based on IgE-mediated targeting.

The current study represents a proof-of-principle for the use of MCs differentiated from human adipose tissue in an allergo-oncological therapy. A significant amount of research is still required to determine the mechanisms of ADMC killing of breast cancer cells and whether or not this technology will function *in vivo*. If successful, however, this technology represents a new strategy for treating tumors through the selective targeting of tumor specific antigens with IgEs that mediate the targeting and release of tumor-killing mediators from MC.

CHAPTER V

FUTURE APPLICATIONS

V.1 Definition of Conditioned Media

One of the most pressing outstanding questions is the exact composition of conditioned mast cell media and more specifically which are the most important components for the purposes of differentiation of MCs. Determining this will not be a trivial undertaking as it is likely to contain many different molecules, only some of which are likely to play a role in the differentiation of ADSCs into MCs. Furthermore, it is possible that all batches of conditioned mast cell media are not identical and determining the degree of batch-to-batch variation will also be important. Ideally, in the future it would be beneficial to use a defined media supplemented with only those growth factors necessary for MC differentiation. Preliminary proteomic studies have been initiated and have produced some results, summarized in **Table 5.1**. These data are intriguing; however, a more exhaustive and comprehensive analysis is likely required that will include the analysis of non-protein-based components of the MC secretome including but not limited to lipids, arachidonic acid derived molecules and nucleic acids. Of particular interest will be the analysis of exosomes secreted by MC and their effect on ADSC differentiation.

V.1.i Haptoglobin

One of the most interesting results from the initial proteomic screen was the presence of haptoglobin. Of the ten proteins identified in the initial proteomic screen, haptoglobin was the third most abundant. Haptoglobin is traditionally understood to be a serum protein that binds free hemoglobin in the blood, however it is increasingly being recognized for its role in immune functions such as B cell differentiation [203], B cell behavior [204], Th2 cytokine production in T cells [205], neutrophil function [206] and the growth of the human MC line HMC-1 [207].

Until very recently haptoglobin was presumed to be produced only by the liver and secreted into the blood. While it *is* of course true that haptoglobin is synthesized in large quantities by the liver, it was determined in 2006 that haptoglobin is *also* produced by human neutrophils and stored in cytoplasmic granules [208]. It was later shown that a form of haptoglobin is produced by leukocytes in mice [203]. It is therefore conceivable that haptoglobin may also be produced by MC despite the fact that studies of haptoglobin expression have as of yet failed to identify MC as a source. This possibility is highlighted by a 2013 study in which the authors conclude that haptoglobin was produced only by the liver, pancreas and bone marrow [209], however skin was not among the tissues examined.

While no data exist showing that haptoglobin plays a role in the differentiation of MC, several pieces of evidence suggest that it could. First and foremost haptoglobin has been shown to bind to MC *in vitro* [207]. While the effect reported was to inhibit growth,

as determined the incorporation of radiolabeled nuclides into the DNA of these cells, the effect of haptoglobin on the differentiation of MC from their precursors remains unexplored. It is also of particular interest that neutrophils both produce, store and presumably secrete haptoglobin [208] and inhibit neutrophil functions [206], showing that in a least one instance haptoglobin can have an autocrine function.

Direct evidence of the interaction between haptoglobin and ADSCs is lacking, however several studies have suggested a connection between the two in the context of allograft tolerance in a rat model [210], [211]. A detailed study of the effects of haptoglobin of the differentiative capacity of ADSCs is needed to determine the relevance of the finding of haptoglobin in conditioned mast cell media.

V.2 *In vivo* Studies

The logical extension of the research performed in this study on the application of ADMC to allergo-oncology will be to perform *in vivo* studies. *In vivo* studies are most commonly carried out first in mice, then in higher organisms. A mouse model of human HER2/*neu* expressing breast cancer has been developed and used to test the ability of anti-HER2/*neu* IgE to decrease tumor burden in mice with humanized FcεRI receptors. This same model will be used to test the ability of anti-HER2/*neu* IgE-sensitized ADMC to locate and kill human breast cancer cells.

V.3 MC Interactions

An important question that remains is how MCs interact with the multitude of cell types they encounter *in vivo* and how these interacts may influence the use of MCs for allergo-oncology. One of the most exciting future directions for the research presented in this study is the investigation of possible interactions between MC and T cells, and the extent to which IgE targeting can play a role. It has been conclusively shown that MC can act as antigen presenting cells (APCs) for T cells [202].

It is thought that the cell type that is ultimately responsible for the majority of cancer cell killing *in vivo* is the T cell. T cell-based therapies are one of the most highly investigated methods of chemotherapy currently under investigation. T cell therapies can be placed in to two broadly defined categories, either monoclonal antibody (mAb) based or adoptive cell transfer (ACT) based [212]. The two most common targets of mAb-based therapies are PD-1 and CTLA4, both of which play roles in checkpoint control of T cell activity. Inhibition of these molecules using inhibitory mAbs has been shown to promote a T cell response that in many cases is able to eradicate the tumor burden. the exact mechanism by which checkpoint inhibition functions remains unknown, however there is some circumstantial evidence that may support a role for MC in this process. The first observation that may support the notion of MC participation in this T cell response is that MC express many of the molecules that are found on antigen presenting cells such as dendritic cells, which are commonly thought of as the cells responsible for presenting

tumor antigens to T cells. These include OX40, CD40 and HLA-DR, the latter two of which are inducible by interferon-γ.

A recently published study describing a local immunotherapy for spontaneous malignancies provides an interesting possible connection between MCs and T cell checkpoint inhibition [213]. In this study, a combination of molecules was used to induce T cell targeting of tumors as well as a type of immunological memory allowing treated animals to respond to subsequent tumor development. The molecules used in this study were a ligand for TRL9, unmethylated CpG DNA and an anti-OX40 antibody. These molecules are interesting when considering the role of MCs in this process as MCs have been previously shown to express both TLR9 [39] and OX40 [214].

Table 5.1 Proteomic Analysis of CMCM Proteins.

Protein	*Pseudonyms*	*Abundance*	*Number of Unique Peptides*	*Function*	*Cellular Localization*	*Tissue localization*	*Reported MC Interactions*
Serum Albumin	ALB, HAS	62.5-86.1%	24	Colloidal osmotic pressure maintenance	Extracellular	Serum - (**Liver)**	Fragments of ALB induce degranulation via MRGPRX2
Apolipoprotein A2	APOA2	37 %	2	Major component of HDL, lipoprotein homeostasis	Extracellular	Serum - (**Liver**, Intestine)	Degraded by MC chymase in mouse models
Haptoglobin	BP, HPA1S	7.50%	4	Serum protein, binds free hemoglobin	Extracellular, granules	Serum - (**Liver**, arteries, lung, adipose, leukocytes)	Inhibits HMC-1 growth, Induced by IL-6
Plasma protease C1 inhibitor	SERPING1	4.80%	3	Inhibits complement protease C1	Extracellular	Serum - (Multiple, **Liver**)	Expressed by BMMCs,
Serotransferrin	Transferrin	0.80%	2	Ferric iron binding, iron transport	Extracellular	Serum - (**Liver**, brain, adipose)	Inhibits histamine production
Inactive phospholipase C-like protein 2	PLCL2, phospholipase C-epsilon 2	0.60%	1	GABA Receptor regulation, Phosphoinositol signaling	Perinuclear	Multiple (27)	None
F-box only protein 50	Non-specific cytotoxic cell receptor protein 1 homolog, NCCRP1	0.30%	1	E3 ubiquitin ligase pathway component	Cytoplasm	**Esophagus**, skin, urinary bladder	None
Ankyrin repeat domain-containing protein 23	Diabetes-related ankyrin-repeat protein (DARP), CARP3	0.20%	1	Glucose metabolism, Skeletal muscle development	Nucleus	Heart, Brown Adipose, **Skeletal Muscle**	None
Protein disulfide-isomerase A2	Pancreas-specific protein disulfide isomerase, PDIp	0.10%	1	Protein folding, estradiol binding	Endoplasmic reticulum	**Pancreas**, GI	None
N-acetylmuramoyl-L-alanine amidase	PGLYRP2, tagL, PGRPL	0.10%	1	Hydrolyzes peptidoglycan	Extracellular	Serum - (**Liver**, testes)	None

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